Universidade de Lisboa

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Departamento de Biologia Vegetal

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Dissertação

MESTRADO EM MICROBIOLOGIA APLICADA

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This thesis was fully performed at the Departamento de Biologia Vegetal, Universidade de Lisboa under the direct supervision of Prof. Dr. Cristina Cruz in the scope of the Master in Applied Microbiology of Faculdade de Ciências, Universidade de Lisboa.

Acknowledgements

I hereby express my sincere gratitude to those who have contributed to the conclusion of this work.

To Doutora Cristina Cruz for accepted me as her student, for her supervision, advice and friendship.

To Marta Delgado, for her friendship, constant advice and support. Her critical spirit really served as inspiration and as a guide for the elaboration of this work.

To Herculana Velez for her support in lab work and for her friendship and life lessons.

To Juliana Melo and Frederico Eutrópio for their friendship, help on the lab and the dynamic discussions about this work.

To all my colleagues, for their friendship and company.

To Jorge Simões for his friendship, patience and love.

To my friends, Maria, Sara, Hélder, Rosa, Bárbara, Juvenal, Joaquim, Maria e Jaciana for their friendship and support through all these years.

To my grandparents, Laura Teixeira and Joel Teixeira for their love and for the values they instilled in me.

To my mother and brother, Vanda Teixeira and Frederico Costa for their love and encouragement. Their determination and courage are an inspiration.

Abstract

Piriformospora indica is a root-colonizing endophytic fungus, belonging to the order Sebacinales (phylum Basidiomycota), that promotes plant growth and confers resistance against biotic and abiotic stresses.

The colonization of barley (*Hordeum vulgare*) roots by this fungus begins with a biotrophic growth phase, followed by a necrotrophic phase in which root cells are actively killed by the fungus.

In this work we subjected the fungus to various C concentrations and sources (glucose and sucrose) in order to understand how C availability and source influence its life strategy.

By varying C/N ratios (but not the C source), two morphologically and physiologically distinct phenotypes were discerned: a “compact” phenotype (low C/N conditions) and an “explorer” phenotype (high C/N conditions).

Our results showed that the compact phenotype of *P. indica* has a higher decomposition potential than the explorer phenotype.

Next, both phenotypes were tested in two hosts: maize (*Zea mays*) and barley (*Hordeum vulgare*). When colonizing the hosts, the phenotypes were indistinguishable and readapted their physiology to their new environments.

Furthermore, both phenotypes were exposed to root exudates and soil extracts (two important elements of the rhizosphere), as well to TiO_2 nanoparticles (which had been suggested to enhance fungal performance) and again physiologically characterized.

All three treatments affected the physiology of *P. indica*’s compact phenotype (low C/N conditions) but did not alter the normal physiology of *P. indica*’s explorer phenotype.

While treatments with soil extracts and TiO_2 nanoparticles exerted a repressor effect on the fungal decomposition potential, exposure to root exudates stimulated the fungus’ ability to decompose N and P.

The functional characterization of *P. indica*’s phenotypes and the understanding of the environmental signals involved in its physiological changes will aid characterization of its functional role in the rhizosphere, and should be particularly useful for the improvement of inoculum production.

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General introduction

Piriformospora indica is a root colonizing endophytic fungi, belonging to the order Sebacinales (phylum Basidiomycota), isolated by Verma and collaborators from the rhizosphere of *Prosopsis juliflora* and *Zizyphus mummularia* plants in Thar Desert, India[1].

The ability of *P. indica* to colonize a wide range of plant species, including crops, and its capacity to grow axenically (unlike arbuscular mycorrhizal fungi-AMF) makes it a very promising organism to improve crop production and quality and to increase nutrient use efficiency[1]. In contrast to AMF, the ability of creating symbiosis with *Arabidopsis thaliana* gives the opportunity for fast and effective study of the molecular basis of fungal–plant interaction.

Piriformospora indica is described to interact with approximately 150 plant species, including agricultural, horticultural, medicinal and other important plants[2].

The fungus has been found to be involved in promoting the growth of several plants, including cereal crops such as rice, barley, wheat and maize, resulting in an enhanced grain yield, higher tolerance to abiotic stress, such as salt and temperature and to increase the resistance to pathogens[3 4 5 6].

Despite the increase in biomass production, when interacting with medicinal plants, the colonization of the roots by *P. indica* led to an increase of secondary metabolites content [7 8].

Not only the mycelium in association with roots but also the culture filtrate of the mycelium has been shown to be effective in seed germination enhancement, growth of the seedlings and also in inhibiting the growth of root fungal pathogens[9]. The culture filtrate of the mycelium contains fungal exudates, minerals, hormones, enzymes, proteins, etc[1 10].

In greenhouse experiments, the effect of *P. indica* culture filtrate was tested in several hosts, such as *Zea mays*, *Brassica campestris, Brassica oleracea* and *Phaseolus vulgaris*. Increases in root and shoot lengths and plant biomass were observed in all *P. indica*-treated hosts [11]. Also, *P. indica* cultures filtrate from TiO_2 treated cultures also has been demonstrated to increase seed germination and growth of broccoli (*Brassica oleracea* var. *italica*) seedlings[11].

Thus, *P. indica* shows an enormous potential to be used as a biological agent for plant growth promotion and plant disease protection.

The ability of the fungus in colonising a wide range of metabolic and morphologically distinct hosts shows that it has a high adaptive capacity, making it an interesting model to understand how the symbiotic lifestyle is influenced by the hosts. For instance, when colonising barley (*Hordeum vulgare*) roots, despite its beneficial effect in the plant, *P. indica* has a biphasic growth, which begins with a biotrophic nutrition followed by a switch to a necrotrophic lifestyle, with the induction of genes related with carbon and nitrogen catabolism[12].

A better understanding of *P. indica* physiological properties will open new directions for biotechnological applications of this versatile fungus, in particular in agriculture.

Objectives

Our aim in this work was to understand how carbon availability in the rhizosphere affects *P. indica* development and life strategy (biotrophic/necrotrophic life styles).

In vitro and *in vivo* experiments were performed in order to characterize *P. indica* physiologically and to understand how the fungus decomposing properties (decomposition potential) are affected in response to different C/N conditions.

In order to previse the behaviour of the fungus in the field, the fungus was also subjected to the presence of two elements of rhizosphere (root exudates and soil extract) at different C/N conditions.

The *in vitro* and *in vivo* study of the physiological characteristics of the fungus will allow the optimization of the parameter of inoculum production and prevision of the fungus behaviour in field application.

Chapter 1: Phenotypic and physiological plasticity of *Piriformospora indica* in response to nutrient availability

Introduction

Fungi are heterotrophic organisms depending on organic matter for their survival [13]. To use the carbon sources available, fungi secret extracellular catabolic enzymes able to degrade molecules with distinct degrees of complexity such as cellulose, lignin, proteins or lipids. In addition, to obtain nutrients for their growth, fungi also provide the products resulting from its degradation action to other microorganisms, and therefore are the primary decomposers of organic material in nature, actively participating in the cycles of C, N, P and other nutrients.

The versatility of fungi in decomposing a wide variety of substrates contributes to their ecological success, and for ecosystem sustainability.

In soil, there are three major functional groups of fungi: decomposers (saprophytic), mutualists and pathogens[14].

Saprophytic fungi can cover all their nutritional requirements exclusively by the decomposition of organic matter, usually they use complex substrates, and therefore play an important role in immobilizing and retaining nutrients from the soil.

Plant pathogenic fungi penetrate plant tissues and decompose them; in order to supply their nutritional needs they may cause plant diseases, nutrient deficiency and occasionally plant tissues death.

Mutualistic fungi are those able to establish a symbiosis in which both partners (the fungus and the host) benefited from the interaction. An example of mutualistic fungi are arbuscular mycorrhizal fungi (AMF) that develop mutualistic associations with plant roots. They colonize plant roots where they help the plant to obtain nutrients such as P and N from the soil. They also have an important role on improving plant resistance to pathogens and increasing plants tolerance to abiotic stresses[15].

All fungi are eukaryotes and their most common body structures are hyphae (filamentous fungi) and single cells (yeasts). The multicellular fungi consist of a network of branched filaments called hyphae. A group or mass of hyphae is called mycelium[13]. Because of its structural organization, hyphae can penetrate deeper in the soil than plant roots, and they are more efficient in absorbing nutrients than plant roots.

The characteristics of the rhizosphere are much dependent on the fungi associated with the roots. For most of the time, rhizospheric fungi are either dormant, or they metabolise and grow very slowly utilising a range of organic molecules. The presence of a living plant dramatically changes

the scenario. In general, the concentration of microbes is higher near to the root surface, where root exudates represent an important source of organic C[16].

The rhizosphere comprises the area of soil immediately surrounding a plant root and represents a highly dynamic environment where complex chemical, physical and biological interactions take place[17]. The structure of the rhizospheric community is not static, since microorganisms are permanently competing for resources. On the other hand, root exudates are also dependent on the microbial community living in the rhizosphere. The rhizospheric microbial community composed by archeae, bacteria, protists and fungi is organized in functional groups, which in equilibrium, contribute to plant health and soil fertility. The strength of the relations between the plant and the rhizospheric microorganisms lies in the importance of their interaction to achieve vital compounds and/or the microenvironment adequate to their lives.

Piriformospora indica

P. indica is a root colonizing endophytic fungi, member of the newly defined order Sebacinales (phylum Basidiomycota), isolated by Savita Verma and colleagues from the rhizosphere of *Prosopsis juliflora* and *Zizyphus mummularia* plants in Thar Desert, India[1]. Due to the characteristics of its symbiosis with plants, *P. indica* was firstly described as a culturable, arbuscular mycorrhizal-like fungus. However, unlike AMF, *P. indica* is not an obligatory biotrophic and can be axenically cultivable on several substrates[1].

The hyphal cells of *P. indica* are thin walled, always hyaline and not pigmented. The dimensions of the hyphae depend on the culture conditions. Under low nutrient conditions, the hyphal cells are very long and thin, whereas when cultivated on complex media the hyphal dimension diameter ranges from 0.6µm up to 3.5µm. The hyphae are regularly septated and they are coiled in several layers, often interconnected by anastomoses. The septa consist of dolipores within the continuous parenthosomes. The parenthosomes are always straight and have the same diameter as corresponding dolipore. Neither clamp connections nor sexual structures are known[18 19] .

P. indica form chlamydospores, that emerge from terminal hyphal tips. The mature spores look similar to the shape of a pear, and their sizes vary between 16 and 33µm in length and 10 and 17µm in width. The spore wall is thicken during the maturing process up to maximum 1.5µm and the initial white color changes to light yellow.

P. indica can be cultivated on several substrates, both in solid or liquid media. When using solid culture media, most of the mycelium grows on agar surface and only few aerial hyphae are formed. The mycelium grows concentric and covers the agar media homogenously. Young mycelium is white, but with age the color changes to cream yellow[19].

In liquid culture media, the colonies are conglomerates of small wadding like globose balls.

Such as with AMF, root colonization by *P. indica* promotes growth, increases plant biomass, host tolerance to abiotic factors[2 3 20 4], and the production of secondary metabolites in medicinal plants[8], and also plays an active role against phytopathogenic organisms, by inducing systemic resistance in plants[18]. Like AMF, *P. indica* has also a wide range of hosts, but unlike AMF it is

able to colonize plants from *Brassicaceae* family, such as *Arabidopsis thaliana.* Similar to AMF, the root colonization by the fungus is strictly limited to the root cortex, where it develops intracellular coils that are different from the arbuscules of AMF[21]. *P. indica* extensively colonizes the differentiation and the root hair zones inter- and intracellulary, while it is rarely detectable in the elongation and meristematic zones[22]. This colonization pattern distinguishes it from ecto- and arbuscular mycorrhizal fungi, which either grow only intercellular or colonize predominantly the deeper cortex layers of younger parts of the root [15].

P. indica develops a symbiosis with plant roots where the fungus benefits from the photosynthetically-derived C produced by the plant. The main form of C exported from the leaves into the roots is sucrose, which is also the main form of C present in root exudates [23]. In order to get C, *P. indica* must produce invertase to cleave sucrose into glucose and fructose (and then import these monosaccharides) or directly import sucrose and hydrolyze it intracellulary.

Endophytic and necrotrophic life style

Regarding the strategies adopted by the fungus to access host nutrients, the relationship between fungi and plant roots can be classified as biotrophic or necrotrophic. Biotrophic association between fungi and roots requires living plants as a source of nutrients, while necrotrophic association implies that fungi kill their hosts and live of the dead tissue.

Root colonization by *P. indica* can be either biotrophic or necrotrophic according to the host characteristics[22 24 25 12]. When colonizing *A. thaliana* plants, the fungus establishes and maintains biotrophic nutrition within epidermal cells. In barley *P. indica* undergoes to a nutritional switch to saprotrophy, despite its beneficial effects on the plant[5 12] .

The switch from biotrophic to necrotrophic behavior of *P. indica* is affected by N availability. At the later stages of barley colonization, *P. indica* genes encoding for carbon and N transporters are strongly induced, namely the high affinity ammonium transporter PiAmt1, at the same time the secretion of hydrolytic enzymes also increase [12]. PiAmt1 displays strong homology (75% aminoacid residues identity) to the high affinity ammonium permease MEP2 from *Saccharomyces cerevisiae*, which is required for pseudohyphal growth induction on N starvation conditions[26] .

Interaction between barley and P. indica

When colonizing barley roots, *P. indica* shows a hemibiotrophic strategy, where the fungus initiates the roots colonization with a biotrophic growth and on the later stages of colonization it undergoes to a necrotrophic nutrition[22 12]. Despite its saprophytic strategy, the presence of *P. indica* on barley led to beneficial effects such as increased biomass and increased tolerance to abiotic stresses. [3]

Cytological studies revealed that on the initial stages of colonization, the fungus grows on the root surface and some hyphae branches penetrate into the intercellular spaces. As the colonization progresses, fungal hyphae pass through rhizodermal cells to subepidermal spaces in maturation zone and at the later stages on colonization the fungus excessively occupies rhizodermal and

cortical cells, resulting in host tissue death. Nevertheless, no root necrosis is detected at the macroscopic level[22 27].

The infection of barley root tissue by *P. indica* displays great heterogeneity regarding tissue age, where the mature parts of roots show higher intracellular growth than the younger zones[22].

Interaction between maize and P. indica

When colonizing maize (*Zea mays*) roots, unlike when colonizing barley roots, *P. indica* establishes and maintains biotrophic nutrition. When hyphae of *P. indica* contact with roots they develop apressoria and the roots are colonized intracellulary at the cortex, where the fungus forms coils and branches[21].

Although maize colonization by *P. indica* is poorly documented by cytological studies, several beneficial effects of the fungus in maize plants are reported by various authors, namely the increase of biomass production and grain yield, and the increase tolerance of maize plants to pathogenic fungi, like *Fusarium verticillioides*[21 6].

Extracellular enzymes

In its transition from a single to a symbiotic life style, *P. indica* has to pass through several steps. The first crucial step for a successful root colonization involves the penetration into the plant roots. The penetration can occur through any weakness in the protecting root tissues or by degradation of those tissues by enzymatic digestion.

The plant cell wall consists of a complex mixture of polysaccharides (cellulose, hemicellulose, xylans) and other compounds secreted by the cell, which are arranged and connected through covalent and noncovalent bonds[23] .

The mechanism of cellulose hydrolysis involves the action of at least three classes of enzymes, endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) Endoglucanases act on the amorphous structure of cellulose, breaking the internal β-1,4 glycosidic linkages, releasing oligossacharides. Exoglucanases catalyze the hydrolysis of external β-1,4 glycosidic linkages, releasing cellobiose from the ends of cellulose chains. Ultimately, β-glucosidases hydrolyze cellobiose and oligossacharides in glucose[28].

Xylans represent the most abundant type of hemicelluloses, and its hydrolysis requires the combined action of several enzymes, including endo-1,4-β-xylanases(EC 3.2.1.8) and β-D-xylosidases(EC 3.2.1.37) [29].

After root penetration, the fungus faces plant protection mechanisms and secretes supplementary enzymes such as esterase, laccase, peroxidase and proteinase[30 31].

Basiewicz performed the physiological characterization of several members of the Sebacinales, including *P. indica* and showed that *P. indica* produces high levels of cellulose and lipase

enzymes and low amounts of pectinase[32]. Moreover, *P. indica* lacks laccase production when cultivated axenically. However, the production of laccase is highly stimulated when *P. indica* is co-cultivated with barley roots [33].

After the establishment of the symbiosis, *P. indica* must be able to deliver nutrients to the plant, mainly N and P. N and P mobilization requires the decomposition of complex organic compounds present in soil, by the activity of proteases and phosphatases enzymes.

P. indica produces significant amounts of acid phosphatases for the mobilization of a broad range of insoluble, condensed or complex forms of phosphate, enabling the host plant the accessibility of adequate P from immobilized reserves in the soil [10].

Among proteases, there are several enzymes that hydrolyze organic matter with different specificities for different amino acid groups [34] [35].*P. indica* has a high level of protease activity[33] .

Strategy

The primary aim of this work was to understand how C source and availability affect *P. indica* development and life strategy (biotrophic and necrotrophic life styles) in monoxenic conditions.

For that propose, we inoculated *P. indica* on Hill-Kaefer broth medium (KM), and changed C availability by adding glucose or sucrose in different concentrations and measured the fungal biomass, since the ability to degrade sucrose is related to the invasive/necrotrophic potential of the fungus [36].

Although there were no differences in the amount of biomass produced by the fungus between glucose and sucrose addition, two different phenotypes were obtained in response to C availability: “Compact phenotype” and “Explorer phenotype”.

The next step of the work consisted in performing the macro-morphological and physiological characterization of each phenotype, in order to understand the factors modulating the fungal morphology. For physiological characterization we measured N and C percentages in the fungus, N ($\delta^{15}N$) and C ($\delta^{13}C$) isotopic signatures, final extracellular pH and the potential activities of some extracellular enzymes involved in organic matter decomposition.

We hypothesized that *P. indica* compact phenotype, expressed at low C/N ratios (C deficiency) has a higher decomposition and colonization potentials than the explorer phenotype.

Next, both phenotypes were applied in two different hosts, maize and barley, whose interactions with *P. indica* are different, since when colonizing maize roots the fungus establishes and maintains a biotrophic lifestyle, but when interacting with barley roots it switches from a biotrophic nutrition to a necrotrophic lifestyle at the later stages of colonization.

Maize and barley also differ on their metabolism, once maize is a C4 plant and barley is a C3 plant. C4 plants may have 50% higher photosynthetic efficiency than C3 plants [37] [23] which may

be reflected on the amount and quality of C exudate through the roots. Thus, on the Maize-*P. indica* system the fungus is possibly receiving more C from the host then in Barley-*P. indica* system, so the decomposition potential of the fungus will be possibly higher when colonizing barley roots, than when colonizing maize roots.

On the other hand, we used two different inocula of *P. indica*, corresponding to the two phenotypes described in the previous chapter, compact and explorer phenotypes. Thus, the compact phenotype (derived from cultures without glucose) has a higher decomposition potential than explorer phenotype (resulted from cultures with 10g.L^{-1} of glucose).

With this experiment we tried to address the question if the inoculum production conditions and the physiology acquired during this process is overlapped by host interference. Thus, if the initial physiological characteristics of the inoculum take precedence over the host-specific influence, plants inoculated with compact phenotype should exhibit higher biomass than plants inoculated with explorer phenotype, since compact phenotype has a higher decomposition potential (and then is more efficient in the nutrient uptake and transfer to the plant). However, if the host influence is a stronger modulator of the fungal physiology than inoculum production conditions, then the inoculated plants should be equally beneficiated, independently of the inoculum applied. Moreover, if this hypothesis is correct, the colonized barley roots should have a higher decomposition potential than maize roots, since barley plants should provide less C to the system than maize plants.

The functional characterization of each phenotype and the comprehension of the environmental signals involved on its physiological changes will give us information about the functional role of *P. indica* in the rhizosphere. Such information can be particularly useful for the improvement of inoculum production.

Materials and methods

P. indica cultivation and culture conditions

P. indica was inoculated into 100mL Erlenmeyer flasks containing 25mL of Hill-Kaefer (KM) broth medium.

KM medium contains several C organic sources (peptone 2g.L^{-1}, casein 1g.L^{-1} and yeast extract 1g.L^{-1}) and glucose (or sucrose), which was added into the culture medium to attain five concentrations (2, 5, 10, 15 and 20g.L^{-1}). A total of 10 different treatments (5 concentrations C * two C sources (glucose or sucrose) with three replicates were tested. The initial medium pH was adjusted to 6.5. Culture media were then autoclaved for 20 minutes at 121ºC and 1 atm.

Since biomass production by *P. indica* was similar using glucose or sucrose, for the experiments of morphological and physiological characterization of each phenotype we used KM cultures with 0g.L^{-1}, 5g.L^{-1} and 10g.L^{-1} of glucose.

The initial inoculum was derived from cultures of *P. indica* with eleven days, grown on solid KM medium. Then, two fully grown fungal agar disks (0.6 cm diameter) were inoculated in Erlenmeyer flasks.

The cultures were maintained at 28ºC, under orbital agitation (200 rpm) for 11 days.

After 11 days of culture, *P. indica* colonies were harvested, washed with sterile distilled water and macromorphologically characterized based on their size, texture, colour, shape and surface as "compact phenotype" or "explorer phenotype".

After 11 days, the cultures were harvested; the fungal mycelium was separated from the culture medium, washed with sterilized distilled water and dried at 50ºC for 48 hours. Data presented correspond to the biomass (mg) per colony.

Measurement of spores

P. indica produced pear-shaped chlamydospores, which stay attached to the mycelium. The spores were dislodged by adding 100 µL of Tween 80 to 10mL of culture broth containing mycelium. The samples were mashed using Omni-Mixer Homogenizer equipment (Solvall®, Norwalk, Conn, USA) during 5 cycles of 3 minutes and sonicated for 5 minutes each (Elmasonix S30, Elma®, Germany), as described by Kumar *et al*. (2010). After their detachment, the spores were stained with lactophenol cotton blue 0.5% (w/v) and measured on microscope (Olympus BX51TF, Japan) using the ocular micrometer.

30 spores per replicate (90 spores for treatment) were measured (fig.1). Concerning the spore's dimension, they were grouped in two classes, small spores (length until 15µm) and large spores (length higher than 15µm).

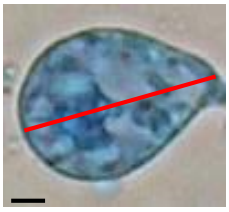


Figure 1.P. indica spore. Spores of P. indica were classified regarding its length (red line), as small or large. Bar 5 µm.

Stable Isotope Ratio Analysis

From each *P. indica* sample, a fragment was cut and lyophilized (Model Alpha1-5, Christ ®). The lyophilized material was milled (Retsch MM 2000, Germany) and approximately 1mg of each sample was weighted for stable isotope ratio analysis.

Stable isotope ratio analyses were performed at the Stable Isotopes and Instrumental Analysis Facility (SIIAF) of the Centro de Biologia Ambiental (CBA), Universidade de Lisboa - Portugal. $\delta^{15}N$ and $\delta^{13}C$ in the samples were determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston and Owens, 1983), on a Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to an EuroEA (EuroVector, Italy) elemental analyzer for online sample

preparation by Dumas-combustion. The standards used were IAEA-N1 and USGS-35 for nitrogen isotope ratio, and IAEA-CH6 and IAEA-CH7 for carbon isotope ratio; $\delta^{15}N$ results were referred to Air and $\delta^{13}C$ to PeeDee Belemnite (PDB). Precision of the isotope ratio analysis, calculated using values from 6 to 9 replicates of laboratory standard material interspersed among samples in every batch analysis, was ≤ 0.2‰.

Percentages of N and C (w/w) were determined using the major mass signal, with L-Cystine OAS and Methionine OAS certified elemental reference materials (Elemental Microanalysis, UK) as calibration standards.

Plant and fungal growth and culture conditions

Seeds of maize and barley were surface-sterilized for 2 min in 70% ethanol solution (v/v) followed by 5 minutes in a NaClO solution (5%, v/v), and finally washed three times with sterile water.

Seeds were germinated on Petri dishes, at 25ºC in the dark. After germination, seedlings were placed in pots containing 600mL of sterile sand each. The sand was sterilized twice at 121ºC for 1 hour, 1 atm.

The plants were grown without the fungal inoculum for 5 days. After 5 days the plants were inoculated with either *P. indica* compact or explorer phenotypes by adding 1mL of inoculum next to the roots. Each treatment had five replicates.

P. indica inocula were cultured on Hill-Kaefër broth (KM) medium with $0g.L^{-1}$ and $10g.L^{-1}$ of glucose (for obtaining compact phenotype and explorer phenotype, respectively), for 20 days at 28ºC, 200rpm. Fungal mycelium was then separated from the culture medium, washed with sterile distilled and dried using filter paper. Mycelium (4g) was homogenised in 40mL of 0.85% (w/v) of sterile NaCl.

Maize and barley plants were grown in a walk-in growth chamber (model 5000EH, Aralab, Portugal) in a controlled environment (photoperiod 16h/8h, 25ºC/20ºC and 70% relative humidity) and fertilized with 25mL of glucose free KM medium once a week. Twice a week the plants were watered with 25mL of tap water.

Plants were harvested 1 month after the inoculation with *P. indica.* Roots were carefully washed with tap water and stored at -20ºC enzymatic assays,

Plant shoots were weighted, dried at 60ºC for 48 hours and again weighted for biomass determination.

Enzymatic assays

From each fungal colony, two fragments were cut and used as samples for enzymatic assays. For the enzymatic assays of the colonized roots three samples were randomly selected from the roots of each of the plants (total of 15 measurements per treatment).

The removed fragments were subjected to measurement of potential activities of the following enzymes: Leucine aminopeptidase (EC 3.4.11.1), β-xylosidase (EC 3.2.1.37), β-glucuronidase

(EC 3.2.1.31), cellobiohydrolase (EC 3.2.1.91), N-acetyl-glucosaminidase (EC 3.2.1.14), β-glucosidase (EC 3.2.1.21) and acid phosphatase (EC 3.1.3.2):

- Leucine aminopeptidase activity indicates degradation of the N-containing compounds.
- N-acetyl-glucosaminidase is an enzyme responsible for chitin degradation.
- Cellobiohydrolase, β-xylosidase, β-glucuronidase and β-glucosidase are involved on cell wall and C-related degradation of organic matter (i.e. involved on organic matter decomposition by the hydrolysis of glycosidic linkages).
- Acid phosphatase hydrolyzes orthophosphate residues from phosphomonoesters under acidic conditions.

All enzyme assays were performed in 96-well filter plates (AcroPrep ™ 96-filter plate with 30–40 lm mesh size; Pall Life Sciences, Crailshelm, Germany). Each sample was subjected to sequential assays of enzymatic activities, as described by Pritsch *et al*. (2011)[38].

Briefly, both samples and blanks were incubated in 150 µL incubation buffer on a microplate shaker at room temperature.

At the end of incubation time of each enzymatic assay, incubation solutions were transferred to measurement plates (black microplates, Nunc, Langenselbold, Germany) using a vacuum manifold and 100 µL of stopping buffer (pH 11) was added.

In between enzyme assays, the samples where rinsed with 150 µL of rinsing buffer [38].

Fluorescence was measured at 360 nm excitation and 460 nm emission using the fluorescence microplate reader (BioTek™ FLx800™).

After the seven enzyme tests, the fungal samples were washed with sterile distilled water, dried at 50ºC for 48 hours and weighted.

Enzyme activities were calculated from fluorimeter readings, as described by Pritsch *et al.* (2011) and were expressed as µmol.h^{-1} of released substrate per gram of mycelium or µmol.h^{-1} of released substrate per gram of root.

C - related organic compounds decomposition and N decomposition potentials corresponded to the sum of all enzymatic activity related to C or N cyling and are expressed as µmol.h^{-1} of released substrate per gram of mycelium or µmol.h^{-1} of released substrate per gram of root. Thus, C decomposing potential corresponded to the sum of the activities of β-xylosidase, cellobiohydrolase, β-glucuronidase and β-glucosidase enzymes. Similarly, N decomposing potential corresponded to the sum of the activities of leucine aminopeptidase and N-acetyl-glucosaminidase. P decomposing potential corresponded to the activity of acid phosphatase, since no other enzymes related to P cycling were tested.

Results

Phenotype description

Depending on the C concentration but not on the source (glucose or sucrose), two phenotypes occurred. Thus, at concentrations higher than 5g.L^{-1} of glucose or sucrose (those used in the culture media) the fungus presented large colonies with rough surface ("explorer phenotype"), while when glucose or sucrose concentrations were lower than 5g.L^{-1}, *P. indica* exhibited smaller, compact and smooth colonies ("compact phenotype") (fig.2). The main macromorphological characteristics of each phenotype are presented in table 1.

In 5g.L^{-1} of glucose cultures the fungal phenotype was not defined, i.e. both phenotypes occurred.

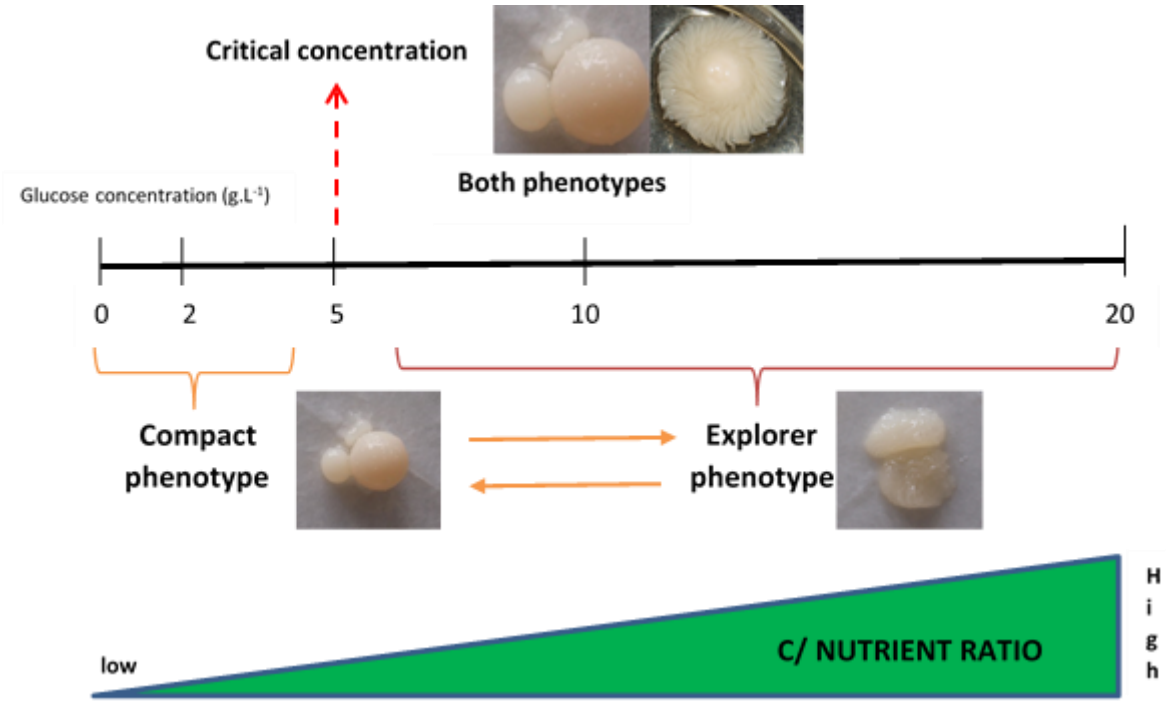
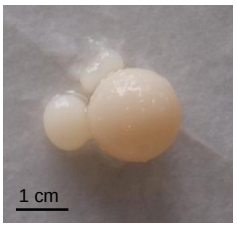
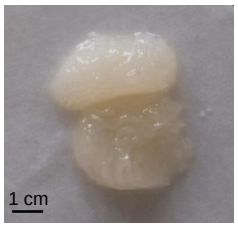


Figure 2. P. indica phenotypes depending on glucose concentration. When incubated on KM medium, for glucose concentrations lower than 5g/L, P. indica always has compact phenotype. For concentrations higher than 5 g/L it always presents explorer phenotype. On 5g/L of glucose, P. indica phenotype is not established, the fungus presents both phenotypes. The concentrations of the other components of the culture medium were constant.

Table 1 Macromorphological characteristics of P. indica compact and explorer phenotypes (N=10).

Characteristics	Compact phenotype	Explorer phenotype
Size	Small	Big
Color	Pale Yellow	Pale Yellow
Shape	Spherical	Fringed
Surface	Smooth	Rough
Texture	Compact	Fluffy
		

Biomass production

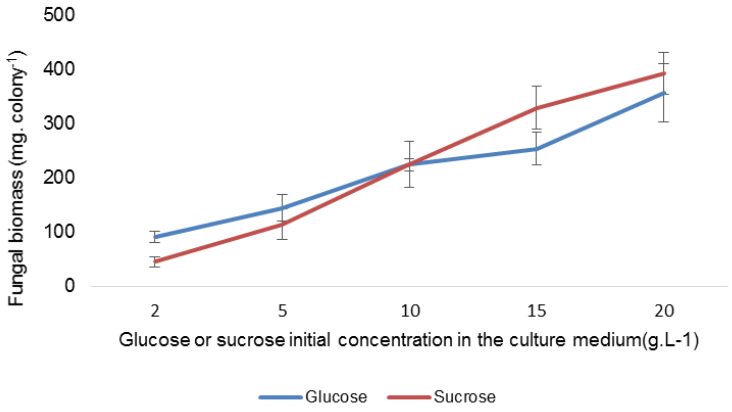


Figure 3.Biomass production of P. indica in 11 days KM cultures with different glucose (blue line) or sucrose (red line) concentrations. The remaining components of the medium maintained the original concentrations. N=15

In both situations biomass production increased with C availability (fig.3).

When glucose was added to the culture medium the colony dry weigh varied between 91 (at 2g glucose. L^{-1}) and 356mg (obtained at 20g glucose. L^{-1}), whereas when sucrose was added the colony biomass ranged from 45 to 392mg.

P. indica tended to growth better with glucose than with sucrose at lowers concentrations and to produce more biomass when subjected to higher sucrose concentration, although the differences were not statistically significant (factorial ANOVA, F=0343, p=0.565).

Spores Proportion

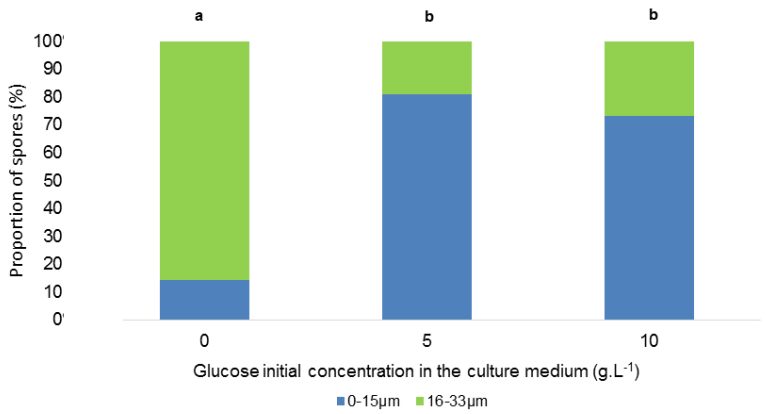


Figure 4. Proportion of small and large spores (blue and green, respectively) of P. indica in response to glucose concentrations. Letters above the bars indicate significant differences ($p<0.05$) accordingly to Tukey test. N=9.

Concerning the proportion of small and large spores, two distinct patterns were obtained in response to glucose availability (fig.4). Thus, 0g.L^{-1} of glucose cultures had higher proportion of larger spores (16-33µm), whereas 5g.L^{-1} and 10g.L^{-1} of glucose cultures had higher proportions of smaller spores (0-15µm).

When glucose was absent from the culture medium 87% of the spores produced by the fungus were small (15µm length) and only 13% showed lengths higher than 16µm (large spores), whereas the culture with the highest concentration of glucose (10g.L^{-1}) produced 27% and 73% of large and small spores, respectively. The patterns of proportion of spores were not altered by glucose concentration (19% of large spores and 81% of small spores at 5g.L^{-1} of glucose).

Nitrogen and Carbon percentages

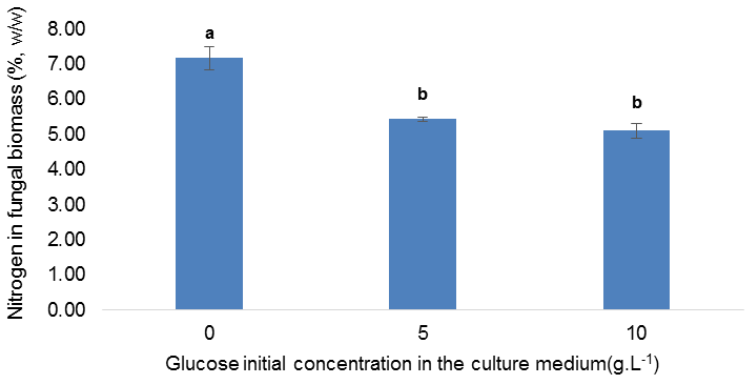


Figure 5.Nitrogen percentage of P. indica colonies subjected to different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Letters above the bars indicate significant differences ($p<0.05$) accordingly to Tukey test. N=9.

Regarding nitrogen percentage, the concentration of N of the fungus decreased with glucose availability (fig. 5). Thus, compact phenotype (resulted from 0g.L^{-1} of glucose culture medium) presented between 6.8 and 7.4% of N, whereas explorer phenotype (resulted from cultures with 10g.L^{-1} of glucose) had between 4.9 and 5.3% of N. In the intermediate situation (5g.L^{-1} of glucose), the proportion of N in the fungus was around 5.4%. The values of N percentage in the fungus when glucose was present in the culture medium (5 and 10g.L^{-1} of glucose) are not statistically different between themselves, but significantly differ from the values on N percentage obtained when glucose was absent from the cultures medium

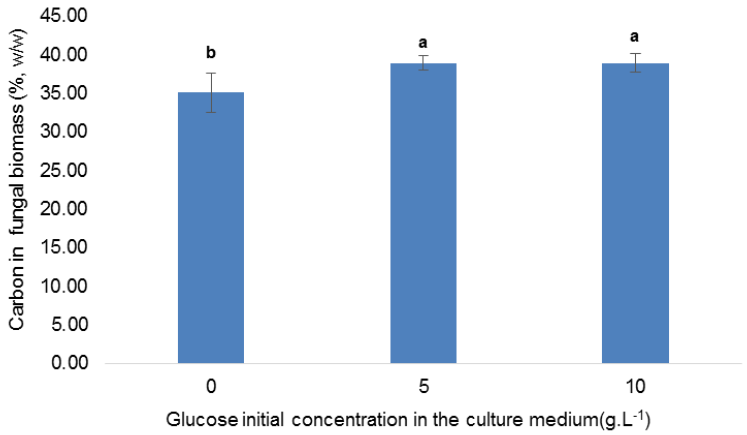


Figure 6.Carbon percentage of P. indica colonies subjected to different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. . Letters above the bars indicate significant differences ($p<0.05$) accordingly to Tukey test. N=9.

Regarding C percentage of the fungus, C percentage increased with glucose availability in the culture medium (fig.6). Thus, the colonies subjected to 0g.L^{-1} of glucose (compact phenotype) accumulated between 32.2 and 36.8% of C, whereas the colonies subjected to 10g.L^{-1} (explorer phenotype) of glucose stored between 37.6 and 39.6% of C. For the intermediate situation (5g.L^{-1} of glucose), the percentage of C in the fungus was between 37.9 and 39.1%. The values of C percentage in the fungus when glucose was present in the culture medium (5 and 10g.L^{-1} of glucose) are not statistically different between themselves, but differ from the values on C percentage obtained when glucose was absent from the cultures medium.

Therefore, the proportion of C increased with glucose availability, so the compact colonies had proportionally less C than explorer colonies.

Stable isotope $\delta^{15}N$ and $\delta^{13}C$ discrimination

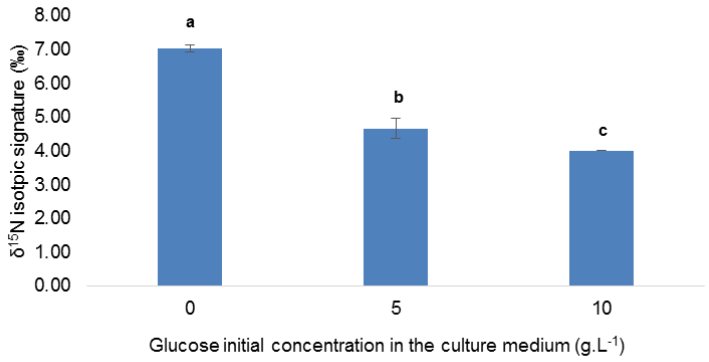


Figure 7. Stable $\delta^{15}N$ isotope signatures of P. indica colonies subjected to different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Significantly different values as revealed by Tukey test are indicated with different small letters ($p<0.05$) N=9.

Table 2. $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures of the culture media and culture media components.

	Isotopic signatures (‰)	
Treatments/ Culture medium components	δ ^{15}N	δ ^{13}C
0g.L^{-1} glucose medium	5.2	-21.7
5g.L^{-1} glucose medium	4.6	-16
10g.L^{-1} glucose medium	4.2	-14.4
Peptone	6.6	-16.9
Casein	6.9	-26.8
Yeast extract	-0.1	-25.5
Glucose	----	-12.1

Each phenotype showed different $\delta^{15}N$ isotopic signatures (fig.7). Thus, compact phenotype (0g.L^{-1} of glucose) showed values of $\delta^{15}N$ higher than the culture medium ($\delta^{15}N$=5.2) and similar to both peptone ($\delta^{15}N$=6.6) and casein ($\delta^{15}N$=6.9), which indicates that it was discriminating for these N sources available in the culture medium (i.e., consuming them preferentially over yeast extract ($\delta^{15}N$=-0.1) as sources of N).

On the other hand, at the intermediate situation, when both compact and explorer phenotypes occur (5g.L^{-1} of glucose) the $\delta^{15}N$ isotopic signature of the fungus ($\delta^{15}N$=4.5), was similar to the isotopic signature of the culture medium ($\delta^{15}N$=4.6) which indicates that on this situation the fungus started to consume also the yeast extract ($\delta^{15}N$=-0.1). Therefore the fungus used all the

sources available in the culture medium (peptone, casein and yeast extract) in the same proportions they were present in the culture medium.

Explorer phenotype (10g.L^{-1} of glucose) also did not discriminate for any of the N sources available, i.e. it isotopic signature ($\delta^{15}N$=4.0) was not identical to neither of the isotopic signatures of the N sources available in the culture medium (peptone $\delta^{15}N$=6.6; casein $\delta^{15}N$=6.9; yeast extract $\delta^{15}N$=-0.1), and was also identical to the culture medium ($\delta^{15}N$=4.2), which indicates that the fungus is consuming all N components of the culture medium on the proportions they were present.

In summary, compact phenotype used preferentially peptone and/ or casein as N sources (over the consumption of yeast extract), whereas explorer phenotype besides peptone and casein also uses yeast extract as source of N (did not discriminate for any of the N sources). Moreover, the consumption of yeast extract increased with the glucose availability in the culture medium.

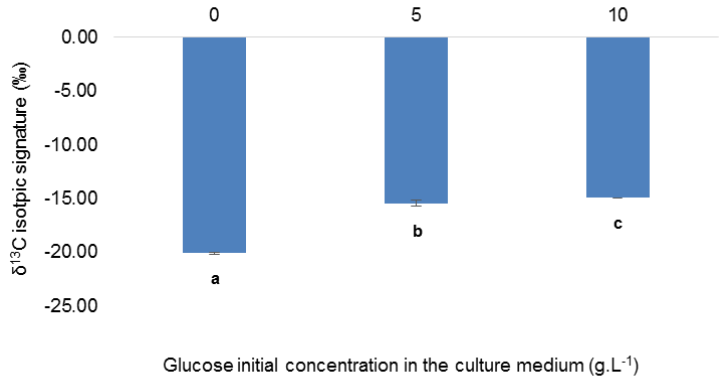


Figure 8. Stable $\delta^{13}C$ isotope signatures of P. indica colonies subjected to different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Significantly different values as revealed by Tukey test are indicated with different small letters ($p<0.05$) N=9.

Concerning $\delta^{13}C$ isotope, each phenotype showed different $\delta^{13}C$ signatures (fig.8).

Thus, compact phenotype has a higher $\delta^{13}C$ isotopic signature than the culture medium (between -20.0 and -20.3) probably because it is consuming the C from the same sources that it incorporates N (peptone and casein) whereas explorer phenotype has $\delta^{13}C$ isotopic signature ($\delta^{13}C$=-14.9) similar the culture medium ($\delta^{13}C$=-14.4), so it is not discriminating for any of the source of C available in the culture medium, i.e. it is consuming all the C sources available in the culture medium (peptone, casein yeast extract and glucose) in the same proportion they were present in the culture medium.

In the intermediate situation (5g.L^{-1} of glucose), fungal isotopic signature ($\delta^{13}C$ between -15.3 and -15.8) was also similar to the culture medium ($\delta^{13}C$ =-16.0), which indicates that the fungus is not incorporating any of the C sources preferentially.

Final pH

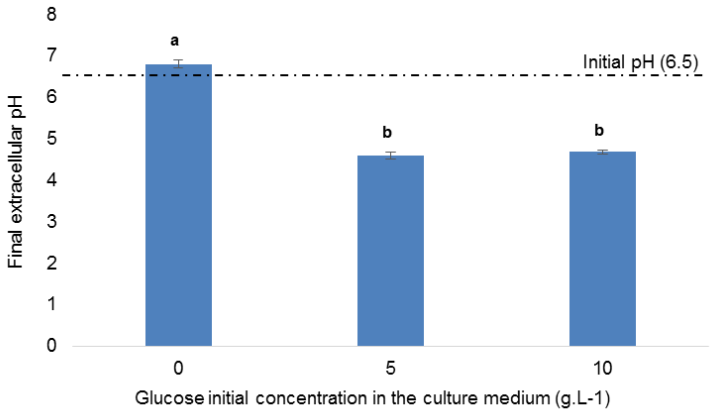


Figure 9.Final extracellular pH of 11 days P. indica cultures subject to different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Significantly different values as revealed by Tukey test are indicated with different small letters (p<0.05) N=9.

Regarding final pH of the culture medium of each sample, the extracellular pH of the compact cultures (0g.L^{-1} of glucose) was higher than the final pH of explorer cultures (10g.L^{-1}of glucose) (fig.9). Thus, when glucose was absent from the culture medium the final extracellular pH varied between 6.66 and 6.89, and when glucose was present at 5g.L^{-1} and 10g.L^{-1} the final extracellular pH of the cultures ranged from 4.51-4.75 and 4.64-4.75, respectively.

In other words, compact cultures led to an alkalization of the original culture medium (initial pH=6.5) and explorer cultures caused an acidification of the culture medium.

Decomposition potentials

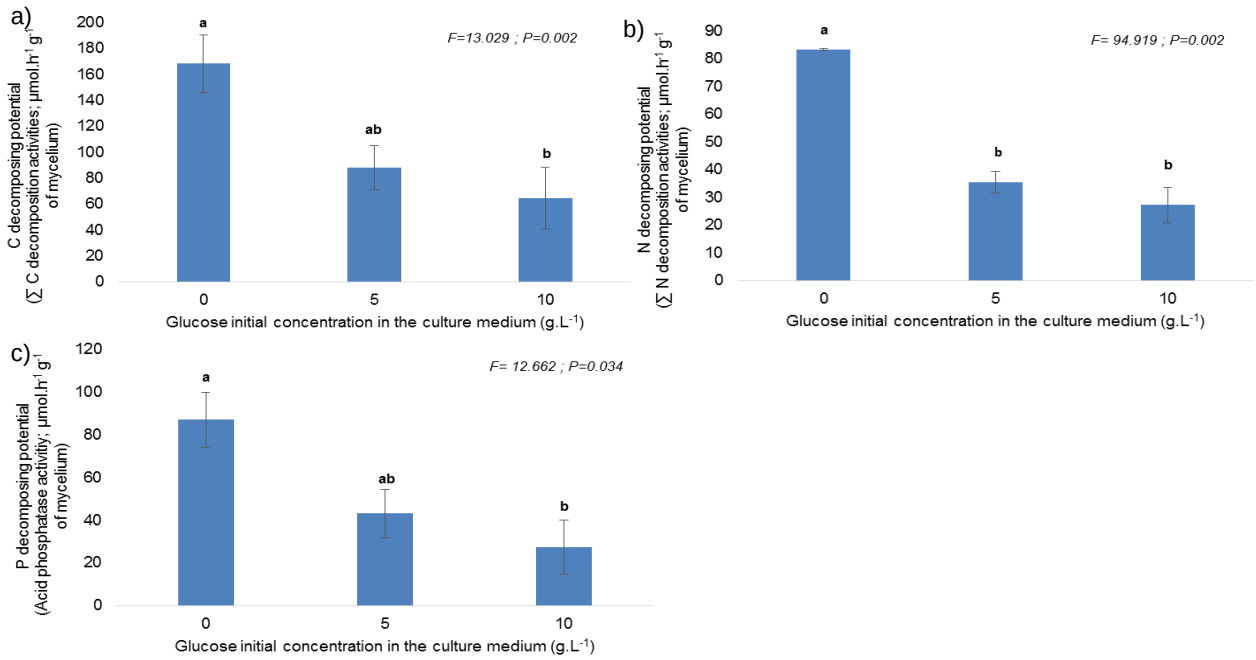


Figure 10.C, N and decomposition potentials (a), b) and c), respectively) of P. indica cultures subjected to different glucose concentrations. The cultures with 0g.L^{-1} of glucose corresponds to compact phenotype; 5g.L^{-1} Compact & Explorer phenotype; 10g.L^{-1} Explorer phenotype. ∑C decomposition: β-xylosidase, cellobiohydrolase, β-glucuronidase and β.glucosidase activities; ∑N decomposition: Leucine aminopeptidase and N-acetyl-glucosaminidase activities; Small letters above bars indicate significantly different values revealed by Tukey test (p<0.05) N=6.

For all nutrient cycling potentials compact phenotype showed higher mean values of enzymatic activities than explorer phenotype (fig.10). Thus, regarding C decomposing potential, compact phenotype exhibited mean value of 168.27µmol.h^{-1}.g^{-1} of mycelium and explorer phenotype present a mean value of 64.75µmol.h^{-1}.g^{-1} of mycelium. On the intermediate situation (5g.L^{-1} of glucose), when the fungal phenotype was not defined, the mean value of C decomposing potential was between the values of the two *P. indica* phenotypes (88.24µmol.h^{-1}.g^{-1} of mycelium).

Concerning N decomposition potential, again compact phenotype had higher values of decomposition activities than explorer phenotype (83.24 and 27.23µmol.h^{-1}.g^{-1} of mycelium, respectively). On the intermediate situation, the mean N decomposing potential of the fungus was very similar to the explorer phenotype (35.42µmol.h^{-1}.g^{-1} of mycelium).

P cycling potential demonstrated the same pattern: compact phenotype showed higher mean values of P decomposition than explorer phenotype (86.96 and 27.18µmol.h^{-1}.g^{-1} of mycelium, respectively). These results suggests that C, N and P nutrient decomposing potentials are negatively correlated with C to N proportions of the culture medium.

Enzymatic activities

Regarding the potential enzymatic activity assays, compact phenotype consistently showed higher values of enzymatic activities than explorer phenotype (fig.11; table 3). Therefore, the absence of glucose in the culture medium stimulated the activity of all catabolic enzymes. These results were expected for the enzymes involved on C compounds decomposition (cellobiohydrolase, β-xylosidase, β-glucuronidase, β-glucosidase and N-acetyl-glucosaminidase), since on 0g.L^{-1} of glucose conditions, the fungal growth is limited by C.

Interestingly, glucose starvation also stimulated the activity of leucine aminopeptidase (activity of 17.54µmol.h^{-1}.g^{-1} of mycelium in compact phenotype and 5.75µmol.h^{-1}.$g^{-1}$of mycelium in explorer phenotype) as well as of acid phosphatase (activity of 86.96µmol.h^{-1}.$g^{-1}$of mycelium in compact phenotype and 21.18µmol.h^{-1}.$g^{-1}$of mycelium in explorer phenotype) enzyme (table 3).

Table 3.Potential enzymatic activities of P. indica phenotypes. N=6

	Potential enzymatic activity (µmol.h-1.g-1 of mycelium) (Mean ± **SD**)						
Phenotypes	Leucine aminopeptidase	β-Xylosidase	β-glucuronidase	Cellobiohydrolase	N-acetyl-glucosaminidase	β-glucosidase	Acid Phosphatase
Compact	17.55 ± **1.67**	19.13 ± **6.87**	1.69 ± **0.08**	36.84 ± **2.29**	65.7 ± **1.11**	110.6 ± **13.11**	86.96 ± **12.92**
Compact & Explorer	8.75 ± **2.49**	8.35 ± **2.42**	2.17 ± **0.97**	15.69 ± **1.47**	26.67 ± **1.48**	62.03 ± **12.20**	43.07 ± **11.29**
Explorer	5.75 ± **2.95**	5.5 ± **2.41**	0.66 ± **0.14**	16.12 ± **5.70**	21.48 ± **3.51**	42.47 ± **15.98**	27.18 ± **12.65**

These results suggest that when glucose is absent from the culture medium the leucine aminopeptidase activity is also related to C mobilization from organic compounds (peptone, casein and yeast extract) which are composed by both C and N.

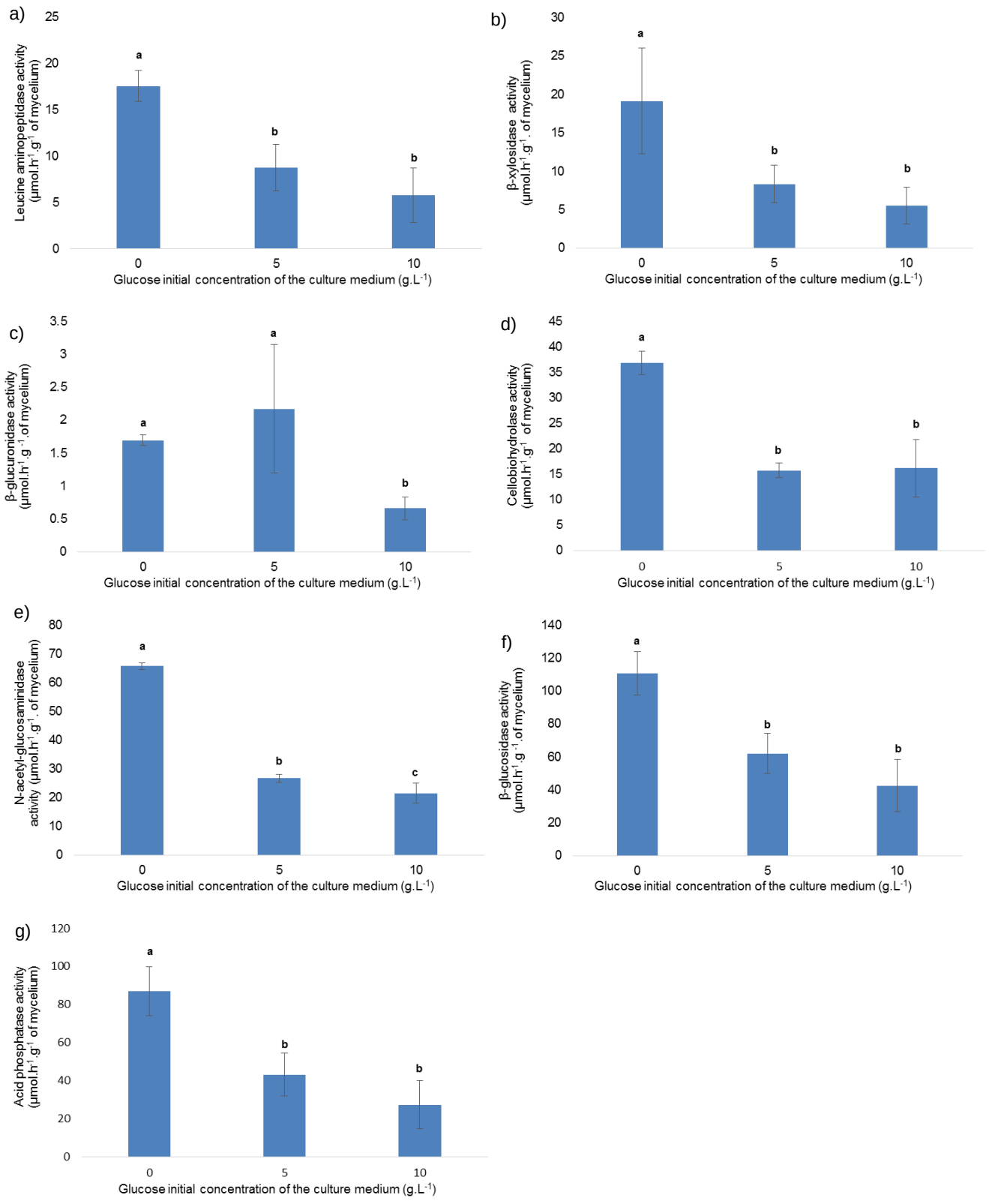


Figure 11.Potential enzymatic activities of P. indica cultures subjected to different glucose concentrations. The cultures with 0g.L^{-1} of glucose corresponds to compact phenotype; 5g.L^{-1} Compact & Explorer phenotype; 10g.L^{-1} Explorer phenotype. a)Leucine aminopeptidase; b) β-xylosidase; c) β-glucuronidase ; d) Cellobiohydrolase; e) N-acetyl-glucosaminidase; f) β-glucosidase; g) Acid phosphatase. *Significantly different values as revealed by Tukey test are indicated with different small letters (p<0.05); N=6.*

These results suggest that when glucose is absent from the culture medium the leucine aminopeptidase activity is also related to C mobilization from organic compounds (peptone, casein and yeast extract) which are composed by both C and N.

The acid phosphatase activity was also higher when glucose was absent from the culture medium, which suggest that the fungus maximize the efficiency of the use of the organic compounds by using the P present on organic sources.

Plant biomass

Table 4. Biomass of maize and barley plants one month after inoculation with P. indica different phenotypes.N=30

Plants	Treatments	Dry weight (g.plant $^{-1}$) (mean ± **SD**)
Maize	Control	1.787 ± **0.233**
	Compact phenotype	1.828 ± **0.134**
	Explorer phenotype	2.035 ± **0.362**
Barley	Control	0.335 ± **0.065**
	Compact phenotype	0.307 ± **0.126**
	Explorer phenotype	0.295 ± **0.123**

Maize plants inoculated with *P. indica* exhibited biomass values between 1.58g.plant^{-1} and 2.39g.plant^{-1} and no inoculated plants had biomass values ranging between 1.55g.plant^{-1} and 2.09g.plant^{-1}.

Barley inoculated plants presented biomass values between 0.19g.plant^{-1} and 0.53g.plant^{-1}, whereas the dry weight of barley control plants varied between 0.23 and 0.40g.plant^{-1}.

For both hosts, the inoculation with *P. indica* did not exert a promoting effect in plant growth (table 4).

Decomposition Potentials

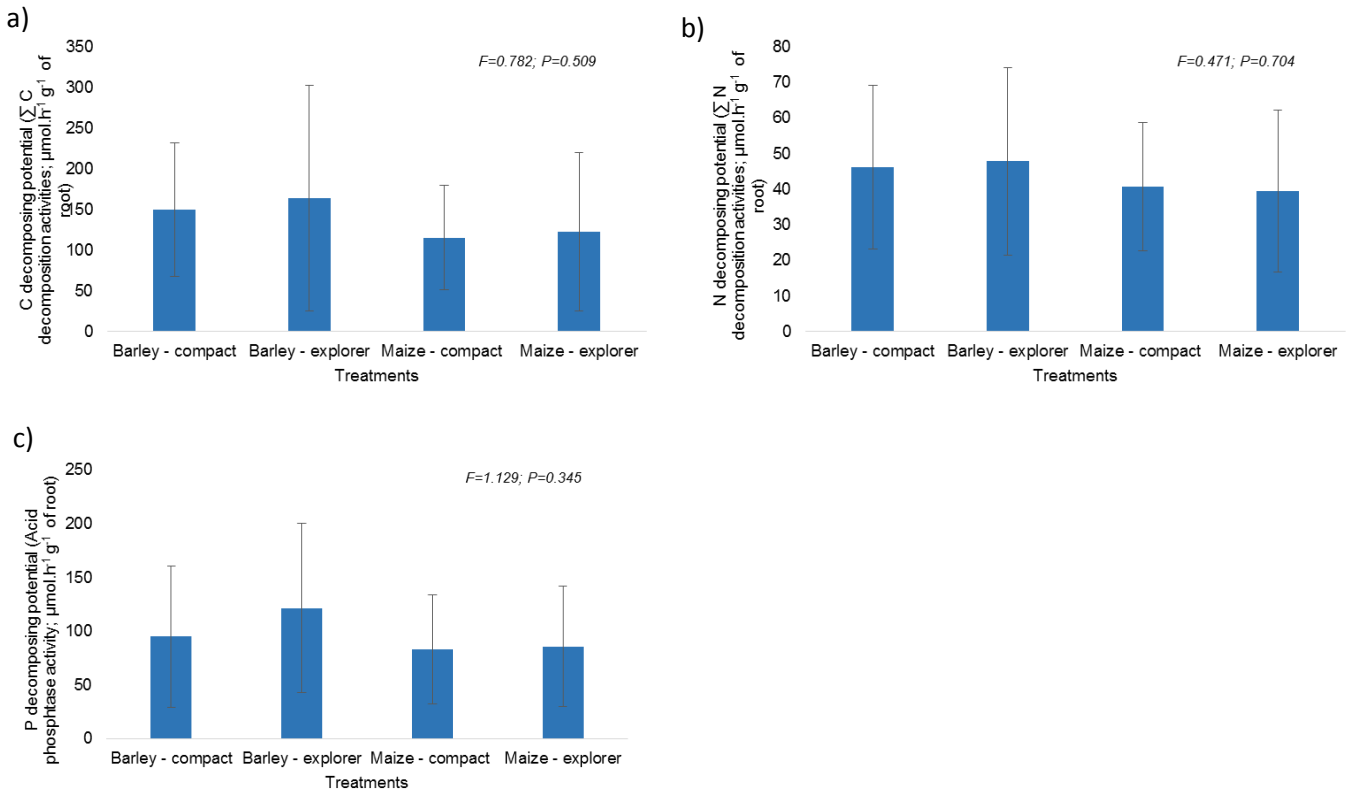


Figure 22.C, N and decomposition potentials (a), b) and c), respectively) l of maize and barley roots inoculated with P. indica compact and explorer phenotypes. ∑C decomposition: β-xylosidase, cellobiohydrolase, β-glucuronidase and β.glucosidase activities; ∑N decomposition: Leucine aminopeptidase and N-acetyl-glucosaminidase activities. N=60.

One-way analysis of variance (ANOVA) was used to determine the differences in organic matter decomposing potentials among the different systems. There were no significant differences in organic matter decomposing potential among *P. indica* phenotytes, nor between the hosts, suggesting that one month after its application on the roots the fungus reflected the host characteristics and not the physiology acquired during the axenical culture (fig.12).

Hence, the decomposing potential of organic compounds (C decomposing potential) by *P. indica* compact phenotype was of 149.92 and 115.45µmol.h^{-1}.g dry root $^{-1}$, and of 163.62 and 122.70µmol.h^{-1}.g dry root $^{-1}$ in explorer phenotype (considering their colonization on barley or maize roots, respectively). Considering N decomposition potential, compact phenotype presented mean values of 46.01 and 40.76µmol.h^{-1}.g dry root $^{-1}$ and explorer phenotype 47.78 and 39.47µmol.h^{-1}.g dry root $^{-1}$ considering their interaction with barley or maize roots, respectively.

Regarding the activity of acid phosphatase (P cycling), compact phenotype presented mean values of 95.09 and 83.02µmol.h^{-1}.g dry root $^{-1}$ and explorer phenotype 121.33 and 85.66µmol.h^{-1}.g dry root $^{-1}$ considering their interaction with barley or maize roots, respectively.

Discussion

Biomass production and phenotype occurrence

Although glucose is described as the preferentially source of C for *P. indica* growth (for concentrations below 10g.L^{-1}) [39] [40]biomass production was similar using glucose or sucrose as C source on KM cultures among the tested concentrations. Similarly, in our experiment, the cultures cultivated with 2g.L^{-1} of glucose tended to have higher biomass than cultures cultivated with sucrose.

Genomic and transcriptomic analyses revealed that *P. indica* can both degrade sucrose extracellulary as well as intracellulary, because it possess both active invertase enzymes and sucrose membrane transporter proteins[25] .

Thus, biomass production increased gradually with the increasing of C concentration in the culture medium, and the maximum production was achieved at 20g.L^{-1} production suggesting that *P. indica* growth was limited by C concentration. Previous studies revealed that on KM culture medium the maximum cell dry weight using glucose as C source is achieved at 40g.L^{-1} [39].

On this experiment, depending on the C concentration but not on the source (glucose or sucrose), two phenotypes occurred. Thus, at concentrations of 20g.L^{-1} of glucose or sucrose (those used in the culture media) the fungus presented large colonies with rough surface ("explorer phenotype"), while when glucose or sucrose concentrations were lower than 5g.L^{-1}, *P. indica* exhibited smaller, compact and smooth colonies ("compact phenotype")(fig.2).

The compact phenotype was firstly described by Suman *et al.* (2010), where nanoparticles of TiO_2 were added to the culture medium, although on that study, they were larger and heavier than explorer colonies [11], implying that the phenotype is independent of the amount of biomass produced.

The occurrence of the different phenotypes can be explained by the need of exploitation of the culture medium by the fungus. Thus, for lower concentrations of glucose there is an excess of the other nutrients. On this situation the fungus uses glucose and the other C sources mainly as energy source and does not invest in the exploitation of the culture medium, as its growth is limited by glucose. Under these conditions the fungus do not expand and form the compact phenotype. However, for higher glucose concentrations, fungal growth is mostly limited by the availability of the other nutrients (mainly N), but it has enough glucose available to invest in biomass. So, *P. indica* modulates its phenotype in order to maximize the uptake of the other nutrients. For that purpose, it expands and forms a fringed surface, which increases the contact area for nutrient uptake from the culture medium.

Proportion between small and large spores

Kumar *et al.* (2011) performed a study in order to determine the optimal culture parameters for inoculum production of *P. indica*. On that study KM culture medium was selected as the optimal for *P. indica* growth and several sources of C and N were tested in order to get a maximum yield of spore production. Their results showed that glucose depletion was a stimulus for sporulation. However, when 20g.L^{-1} or less of glucose was added initially to the medium, it was consumed by the end of exponential phase of growth (between days 3 and 5), chlamydospores started to appear just one day after glucose exhaustion and sporulation process continued for more three days [39].

Considering that the compact phenotype results from cultures without any glucose and explorer phenotype results from cultures with 10g.L^{-1} of glucose, the differences of the sizes of the spores of each phenotype (fig.4) can be explained by the delay in the start of the sporulation process when glucose was present in the medium. Thus, compact phenotype started to sporulate earlier in response to glucose starvation, so the spores produced had more time to mature and grow than the spores that were produced by explorer phenotypes (produced 3-5 days after).

Moreover, since large spores also can be classified as mature and small spores as young[19], we can conclude that in absence of glucose (compact phenotype) the fungus invested more in dispersing structures than in vegetative growth when compared to the cultures grown with glucose.

Nitrogen and carbon percentages

Concerning N proportion in the fungus, it decreased with glucose concentration (fig.5). Also, the percentage of C in fungal biomass was altered by glucose availability, and compact phenotype (0g.L^{-1} of glucose) showed lower concentration of C than explorer phenotype (10g.L^{-1} of glucose) (fig.6). Thus, cultures without glucose showed that the availability of N in the culture medium was excessive: the fungus (compact phenotype) had an excess of N available to incorporate into its biomass and also the proportion of N in fungal dry matter (biomass) was excessive. However, as explorer phenotype which grown in presence of glucose (10g.L^{-1}) had more C available to incorporate into its biomass (that was higher than the biomass of compact phenotype), but the same amount of N available, the N proportion in fungal biomass was lower than in compact phenotype.

As expected, C concentration was higher in explorer phenotype than in compact phenotype, since explorer phenotype has glucose available in the culture medium, which is easier to metabolize C source than peptides and aminoacids present in the complex organic sources of the culture medium (peptone, casein and yeast extract, and exclusive sources of both N and C for compact phenotype).

Stable isotope $\delta^{15}N$ and $\delta^{13}C$ discrimination

The stable $\delta^{15}N$ and $\delta^{13}C$ signatures confirmed that *P. indica* morphology is modulated by the proportion between C and N and not directly by glucose availability.

Thus, compact phenotype (0g.L^{-1} of glucose cultures) used the same organic sources (peptone and casein) in order to get both N and C, and did not use much yeast extract (figs 7 and 8).

On the other hand, explorer phenotype (10g.L^{-1} of glucose cultures) consumed the N sources of the culture medium ($\delta^{15}N$ isotopic signature identical to the culture medium), which is a signal of N starvation. The same phenotype has a similar $\delta^{13}C$ isotopic signature of the culture medium, which indicates that it is taking the C from all the sources available in the culture medium. On those conditions the fungus is probably using glucose as main source of C (since it is the easily metabolized) but it is also taking C from one or more of the organic sources that is using to get N.

Therefore we can conclude that compact phenotype is resulting from C limiting and N excess conditions and explorer phenotype occurrence is due to N starvation and C excess in the culture medium. So the C to N proportion is the signal for the morphological switch of *P. indica*.

On *S. cerevisiae* the transition from buddy to pseudohyphal growth in response to N starvation is well documented. In *S. cerevisiae* the high affinity ammonium transporter MEP2 assumes a central role on morphology modulation. Thus, on N starvation conditions, the expression of this transporter led to the activation of the genes involved in filamentous growth, via cAMP/PKA and MAPK cascade pathways[41 42 43 44].

Since the major differences between compact phenotype and explorer phenotype are related to N metabolism (N percentage and stable $\delta^{15}N$ isotope signature), we think that the phenotype plasticity of *P. indica* can also be regulated, at least partially, by PiAMT1 gene expression.

Final pH

In absence of glucose in the culture medium, the *P. indica* growth lead to an alkalinization of the medium, whereas cultures where glucose was initially added (explorer phenotype) lead to an acidification of the culture medium (fig.9).

Extracellular pH can be considered as an indirect measurement of growth and overall cellular metabolic activity, because the process of C catabolism, from which the cell gets the essential energy for growth, also results in several organic acids that are excreted from the cell (acidifying the extracellular environment). Therefore medium acidification is taken as proxy of growth[45]. However, alkalinization of the medium by glucose-starved eukaryotic cells has also been described by several authors [46 47 48 49]. The main reason for the alkalinization of the medium of compact phenotype cultures is the release of ammonia, a strong base, from the cells due to the use of aminoacids as C source[49].

Potential Enzymatic activities

On our study we simulated free-living and symbiotic stages of *P. indica* by removing or adding glucose to the culture medium. Although the main form of C delivered by the plant to the fungus is sucrose, *P. indica* is capable to cleave those sucrose into glucose and fructose, so when colonizing a host it has more glucose available then when on a free lining stage. Thus, when glucose is absent from the culture medium (simulation of free-living stage) the fungus must be able to use the other organic compounds present in the culture medium in order to get both N and C, but when glucose is present (simulating the glucose that the fungus has available when on a symbiotic stage) the major limiting nutrient is N.

Hence, as we hypothesized *P. indica* compact phenotype (that occurs at low C/N conditions, with C starvation) has a higher decomposition potential than explorer phenotype.

For all tested enzymes the compact phenotype showed consistently higher values of enzymatic activities than explorer phenotype (fig.11). However, the differences in enzymatic activities between $5gL^{-1}$ and $10g.L^{-1}$ of glucose cultures were not statistically significant. Therefore, when *P. indica* has only complex organic sources available in order to get C it was expectable that C related enzymes (i.e. enzymes that directly cleave glycosidic linkages) to have a higher activity than when it has glucose available in the culture medium. However, for leucine aminopeptidase this pattern was not expectable, since on $0g.L^{-1}$ of glucose conditions, there was an excess of N in proportion to C so the activity of this enzyme should be lower than at $10g.L^{-1}$ glucose situation, where N became the limiting nutrient. These results suggest that leucine aminopeptidase activity is also related with C mobilization and consolidate the previous results that shown that when glucose is absent from the culture medium *P. indica* selects the N sources taking in account the amount of C present on each of the organic sources.

Like for leucine aminopeptidase, acid phosphatase higher activity on compact phenotype was not expectable, since KH_2PO_4 is available in the culture medium. However, when the proportions of complex organic sources in the culture medium are higher (at $0g.L^{-1}$ of glucose concentration) the fungus choose to integrate the P present in the organic sources instead to use the KH_2PO_4, in order to maximize the use of the organic sources available. When glucose is present in the culture medium, *P. indica* uses glucose as preferential C source (instead the other organic compounds) and uses the inorganic source of P (KH_2PO_4) available.

Similar results have been reported by several authors where the addition of organic waste materials increased microbial acid and alkaline phosphatase activities in soil [50] [51].

Lahrmann *et al*., recently show that the necrotrophic behavior of *P. indica* on later stages of colonization of barley roots is associated to the induction of the fungal genes encoding for hydrolytic enzymes and C and N transporters, namely the high affinity ammonium transporter PiAMT1 (MEP2 homolog), which plays a central role in the regulation of dimorphic growth in *S. cerevisiae*[12].

The differences between our results and those described by Lahrmann *et al.* can be justified by the different phases of the fungus life cycle. In our experiments, although our aim was to mimic the interaction of the fungus with the plant by adding glucose to the culture medium, the fungus was grown axenically (so in a free-living stage), not being subjected to the other nutritional and chemicals signals that usually are present in root exudates, as sucrose, aminoacids and phenolic compounds. However, on Lahrmann *et al.* experiment the fungus was on a symbiosis stage, so it was never limited by C availability, as well it was exposed to all chemical compounds present in root exudates that besides the nutrients availability also act as physiological regulators in the fungus.

Application of P. indica phenotypes in vivo

The application of *P. indica* inocula in maize and barley roots did not exert a visible beneficial effect on hosts biomass after one month (table 4), which highly contradicts the results referred in the literature[4 40 52] .

The main reason for these results can possibly be the type and the amount of nutrition added to the plant (KM glucose free medium). We think that the rich nutrition allowed the growth of other microorganisms able to decompose the organic compounds present in KM medium leaving nutrients available to plant.

Also, although the main hydrolytic activity in the system might be due to microbial activity, plant roots also have their own enzymatic machinery, which is capable of degrade organic matter [53].

Thus, for being on a nutrient rich environment the hosts did not directly benefit from the action of the fungus.

We compared the decomposing potential of *P. indica* compact and explorer phenotypes colonizing maize and barley roots by the analysis of the decomposition potential of the enzymes related to organic matter decomposition (C, N and P cycles) (fig .12). Since maize is a C4 and barley C3 plant, maize can provide more C than barley to the fungus. As we concluded, *P. indica* compact phenotype (cultured at lower C/N ratio) has a higher decomposition potential than *P. indica* explorer (cultured at higher C/N conditions) we suggest that the fungal phenotype at the time of colonization could determine the decomposition potential of the colonized roots of both barley and maize plants.

However, contradicting our hypothesis, the decomposition potentials were not significantly different among P. *indica* phenotypes neither among the hosts (fig.12). These results can be explained by the great heterogeneity of the data. In fact, there was a wide range of enzymatic activities, which can be due to the different levels of colonization of the roots, since the root samples were chosen randomly. Also different areas of the colonized roots can have different decomposition potentials. Biotrophic and hemibiotrophic fungi have been shown to induce plant N mobilization and accumulation at the side of infection. In particular, N-rich aminoacids such as glutamine and asparagine have been identified as the major forms of N reallocation during infection of different hosts[54 55]. Also, the interaction of *P. indica* with barley roots led to induction

of plant N mobilization to the younger part of the roots, causing the switch for necrotrophic behaviour of the fungus in the basal region of the roots[12].

These results suggest that after one month under the influence of the host, the fungal physiology is adapted to the new environment and that both hosts provided similar amounts of C to the fungus. In fact, plant growth conditions (photoperiod 16h/8h, 25ºC/20ºC and 70% relative humidity) were not ideal for maize plants to have an enhanced photosynthetical performance in relation to barley plants, since the optimal photosynthetically yield of C4 plants is achieved at high temperatures (above 30ºC) or at high light densities[23 56].

Conclusion

On this study we characterized morphologically and physiologically two phenotypes of the fungus *P. indica* (compact and explorer phenotypes). The occurrence of each of the phenotype is regulated by the proportions of C to N in the culture medium and is independent of the C source (glucose or sucrose).

Besides the macromorphological differences between both phenotypes, they also were physiologically distinct. Our results revealed that the compact phenotype has a higher decomposition potential (necrotrophic lifestyle) than explorer phenotype (biotrophic lifestyle), so the glucose depletion in the culture medium was a stimulus for the secretion of organic matter hydrolyzing enzymes. Therefore, our results suggest that *P. indica* phenotypic and physiological plasticity is modulated by the C to N proportions in the environment, and depending on the phase of its life the fungus presents different nutritional demands. Thus, when on a free living stage, glucose depletion is a "stronger" signal than N starvation for the fungus to increase the secretion of hydrolytic enzymes. Nevertheless, during symbioses, when C is supplied by the plant, N starvation is the major stimulus for saprophytic behavior of *P. indica* (as described by [12]). To verify this hypothesis further studies are required.

By having a higher potential of decomposition, *P. indica* compact phenotype should be more efficient in the transfer of soil nutrients for the roots, so it must exert a higher beneficial effect in it host. However, when both phenotypes were tested *in vivo* there were no differences in the decomposition potentials among the fungal phenotypes or the hosts, suggesting that the fungus readapted it physiology to the new environment (presence of a host) and the hosts supplied similar amounts of C to the fungus.

In conclusion, the phenotypic and physiological plasticity of *P. indica* establishes a highly adaptive ability in response to different environmental signals. Depending on the environmental conditions, namely on the proportion of C to N, the fungus adapts it morphology as well it physiology, revealing its functional versatility in the rhizosphere.

Further studies are needed in order to test the different *P. indica* phenotypes in more extreme conditions, where the hosts will really benefit of the potential of each phenotype, to understand if the characteristics acquired *in vitro* will persist on field conditions.

Chapter 2: The influence of root exudates, TiO_2 nanoparticles and soil extract on *Piriformospora indica* physiology

Introduction

Root exudates

Plant roots release a wide range of compounds, which are involved in complex food webs and communication processes in the rhizosphere. These compounds include sugars, polysaccharides, amino acids, aromatic acids, fatty acids, sterols, phenols, vitamins, plant growth regulators and other secondary metabolites that are broadly referred to as root exudates [57] [58] [59].

There are numerous factors that influence the composition and release of root exudates, such as plant age, physiological state, soil composition and soil microbial community itself.[60]

On the other hand, root exudates also play an active role in the modulation of the rhizospheric microbial community[16]. Signalling molecules present in root exudates are essential in the modulation of plant-microbe interaction, since they can act as chemical attractants or repellents of microorganisms[61]. For example, flavonoids act as chemoattractants for rhizobia bacteria and as specific inducers of nodulation genes (*nod*-genes), and strigolactones are responsible for hyphae branching in arbuscular mycorrhizal fungi, which is thought to be essential for the establishment of the symbiosis between the fungus and the plant.[62]

Root exudates have been shown to accelerate the germination of the spores of *Glomus intraradices*, and to stimulate the catabolic metabolism of germinating spores by an increase in mitochondrial biogenesis and the upregulation of genes related to mitochondrial activity. [63] [64]

Transcriptomic analysis of the root colonizing bacterium *Bacillus amyloliquefaciens* FZB42 revealed that three major functional gene groups are strongly stimulated in response to maize root exudates: genes related to N and C uptake and catabolism, genes related to the sensing, motility and biofilm production, and genes related to the synthesis of secondary metabolites with antimicrobial action.[65]

Nanoparticles of TiO_2 as regulatory molecules

Nowadays, nanomaterials are used in many science fields, such as chemistry, biology, physics, materials science, and engineering. In recent years nanomaterials have received a lot of attention and concern due to their fast increasing applications in various areas of economical relevance, such as electronic, pharmaceutics, textiles, cosmetic and environmental remediation[66]. Due to the development of nanotechnology, it is extremely important to determine the effect of nanoparticles on the environment and health. For that purpose, the best models to use are the biological systems[67].

TiO_2 nanoparticles are reported to induce spinach seed germination and plant growth and to increase biomass, chlorophyll synthesis and metabolism in other photosynthetic organisms. These positive effects may due to the antimicrobial properties of nanoparticles, increasing the strength and tolerance of the plants to stress. TiO_2 is also reported as a metabolic regulator, involved in glucose uptake and flux of whole glycolysis pathway in *Escherichia coli*, *B. subtilis* and *S. cerevisiae*[68].

The addition of TiO_2 nanoparticles to *P. indica* cultures results in the increase of fungal biomass and colony diameter. The morphology of fungal colony is also altered from a colony with rough surface to a smooth, bigger and shiny colony (corresponding to the compact phenotype)[11]. Further, the TiO_2 nanoparticles treated *P. indica* culture filtrate has also shown to be helpful in seed germination and growth of broccoli (*Brassica oleracea* var. *italica*) seedlings.

Soil extract

Soils have very distinct microbial communities which are the result of many different factors, including the physical and chemical characteristics of the soil (e.g., soil texture, nutrient and organic matter content and pH) and environmental factors such as climate and vegetation [69] [70].

Soil mineral particles have different chemical composition and surface properties that influence microbial survival and activity and soil solution composition.

Soil also contains organic debris from plant, animal or microbial origin (soil organic matter) with a wide range of chemical properties and potential for biological decomposition [71]. Soil organic matter then represents a major source of C, N, P and sulphur to both plants and microbes in the ecosystems. Fungi, by their physiological versatility, are an advantage to plants since they are able to produce several hydrolytic enzymes that synergistically decompose a wide range of complex substrates.

There are not studies about the influence of soil extract in *P. indica* physiology. However, a transcriptomic analysis of the root colonizing *B. amyloliquefaciens* FZB42 strain revealed that the addition of soil extract to the culture medium led to a repression of the enzymes related to carbohydrate metabolism[65].

Our aim by adding soil extract to *P. indica* cultures was to simulate in part the natural experience of the fungus in the soil rhizosphere, evaluating the role of soil solution as a regulatory agent of *P. indica* metabolism.

Strategy

In this experiment the different phenotypes of *P. indica* characterized on the first chapter were subjected to three different treatments, root exudates, TiO_2 nanoparticles and soil extract, in order to assess the impact of these factors on the fungus physiology.

Since that on the previous chapter the results about the application of *P. indica* phenotype *in vivo* on different hosts were inconclusive, we decided to study the individual influence of two important elements of rhizosphere (root exudates and soil) on fungus physiology *in vitro*.

Besides being an additional source of C and N, root exudates contain several low molecular weight compounds that mediate the communication between the plant and microorganisms in the establishment of the symbiosis and then modulate their metabolism.

By adding root exudates to the culture media, we intended to activate the metabolic machinery implicit on the transition process from a free-living to a symbiotic stage of *P. indica.* Thus, it is expected that the activity of the enzymes involved in the colonization process to be stimulated.

To simulate in part the conditions that *P. indica* experience in rhizospheric soil, soil extract was added to the culture media.

Also, since TiO_2 nanoparticles-treated *P. indica* culture filtrate is a commercialized product used for the growth of economically important plants in the field, but a physiological approach about the effect of TiO_2 nanoparticles in the fungus has not been done, we decided to test their influence on fungal physiology.

Previous studies reported the role of TiO_2 nanoparticles in the enhancement of biomass of *P. indica* and of fungal morphology to bigger and smoother colonies[11]. Furthermore, an independent test was performed with the culture filtrate of the TiO_2 treated fungal broth, in which the seed germination of broccoli (*B. oleracea* var. *italica*) and plant growth was stimulated. However it remains unclear how TiO_2 nanoparticles act on *P. indica* metabolism.

Materials and Methods

Piriformospora indica and culture conditions

P. indica was inoculated into 100mL Erlenmeyer flasks containing 25mL of Hill-Kaefer (KM) broth medium, at three different glucose concentrations (0, 5 and 10g.L^{-1}). The initial pH of the culture media was adjusted to 6.5 and media autoclaved for 20 minutes at 121ºC and 1 atm. After cooling, root exudates or soil extract were added to the culture media. TiO_2 nanoparticles were added to the culture medium before autoclaving. Each treatment had three replicates.

According to the treatments the KM broth medium was supplemented with: sterile maize root exudates at a final concentration of 25% (v/v); sterile soil extract in a final concentration of 10% (v/v); and TiO_2 nanoparticles at a final concentration of 100mg.L^{-1}

The initial inoculum was derived from cultures of *P. indica* with eleven days grown on solid KM medium. Then, two fully grown fungal agar disks (0.6cm diameter) were inoculated on Erlenmeyer flasks of 100mL. Cultures were maintained at 28ºC and under orbital agitation (200rpm) for 11 days.

Maize hydroponic culture and root exudates collection

Maize seed were surface sterilized in 70% ethanol (v/v) for 3 minutes and in 5% sodium hypochlorite solution (v/v) for 3 more minutes and then washed with sterile distilled water.

Seeds were germinated in Petri dishes at 25ºC under dark conditions for 3 days. The germinated seeds were transferred to autoclaved tubes containing 10mL of sterile water (1:1 distilled water and tap water, v/v), with the maize seeds being placed just above the water surface.

The tubes were kept under sterile conditions and maintained in a plant growth chamber (photoperiod 16h/8h, 25ºC/20ºC and 70% relative humidity) for 15 days.

The root growing media (referred to as root exudates) was collected every 3 days and the tubes were refilled with sterile water. Root exudates were sterilized by filtration, using a 0.22µm sterile filter and stored at 4ºC until use.

Soil extract and TiO_2 nanoparticles preparation

Soil extract was prepared with 500g of dried fertile garden soil mixed with one litter of distilled water, followed by stirring at room temperature for 2 hours and autoclaving. After cooling, soil extract was filtered through a 0.22µm filter and then stored at 4ºC until use.

TiO_2 nanopowder (Sigma-Aldrich®) containing TiO_2 nanoparticles (particle size <25nm) was dissolved on KM broth medium at a final concentration of 100mg.L^{-1} before sterilization.

Stable Isotope Ratio Analysis

From each sample, a fragment was cut and lyophilized (Model Alpha 1-5, Christ®). The lyophilized material was milled (Retsch MM 2000, Germany) and approximately 1mg of each sample was weighted for stable isotope ratio analysis. Stable isotope ratio analysis methodology was performed as the described on the previous chapter.

Enzymatic assays

From each fungal colony, two fragments were cut and used as samples for enzymatic assays.

The removed fragments were subjected to measurement of potential activities of the following enzymes: Leucine aminopeptidase, β-xylosidase, β-glucuronidase, cellobiohydrolase, N-acetyl-glucosaminidase, β-glucosidase and acid phosphatase.

All enzyme assays were performed as described on the first chapter.

Enzymatic activities were calculated from fluorimeter readings, as described by Pritsch *et al.* (2011) and are expressed as $\mu mol.h^{-1}.g^{-1}$ of mycelium.

C-related organic compounds (C), N and P decomposition potentials definition is the same as described on the first chapter.

Results

Nitrogen and carbon percentages

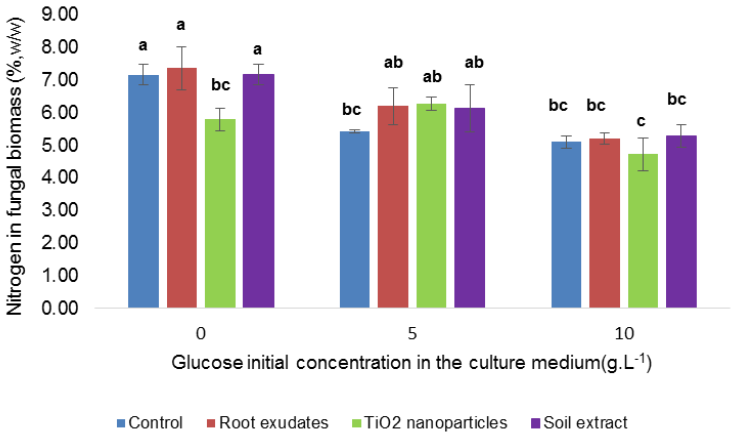


Figure 13. Nitrogen percentage of P. indica colonies subjected to root exudate (red bars), TiO_2 nanoparticles (green bars) and soil extract (purple bars) treatments at different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Letters above the bars indicate significant differences ($p<0.05$) accordingly to Tukey test. N=36.

Concerning N proportion in the fungus, all treatments showed a similar pattern to the control, where the N percentage decreased with glucose availability, except for the treatment with TiO_2 nanoparticles (fig.13). Thus, for the control, root exudates treatment and extract soil treatment, there was a decrease in N concentration with glucose availability with compact phenotype showing higher percentages of N (around 7.1%) than explorer phenotype (around 5%). In the intermediate situation (5g.L^{-1} of glucose) the fungal N percentage was not different of neither of compact phenotype or explorer phenotype.

For 0g.L^{-1} glucose, when TiO_2 nanoparticles are added to the culture medium, the fungal N percentage, decreased from 7.1% to 5.8%. For the same treatment at 5g.$L^{-1}$of glucose the percentage of N increased from 5.4% to 6.2%.

At 10g.L^{-1} of glucose the cultures subjected to TiO_2 nanoparticles treatment showed similar values of N percentage to the control (around 5%).

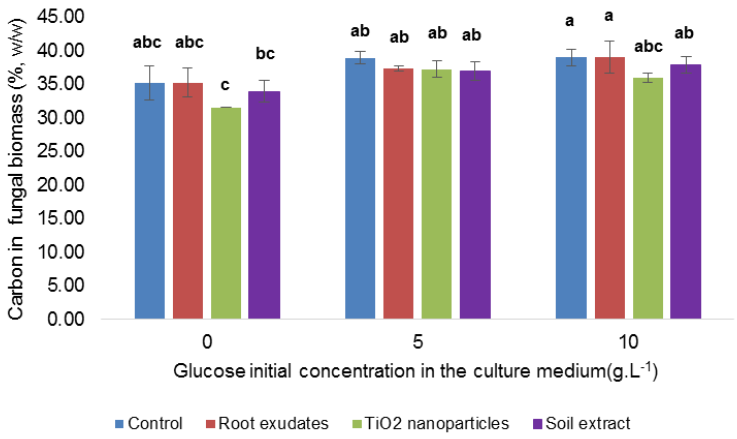


Figure 14. Carbon percentage of P. indica colonies subjected to root exudate (red bars), TiO_2 nanoparticles (green bars) and soil extract (purple bars) treatments at different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Letters above the bars indicate significant differences ($p<0.05$) accordingly to Tukey test. N=36.

Regarding the fungal C percentage, again only the TiO_2 nanoparticles treatment had affected the accumulation of C by fungus, although this effect was only visible at $0g.L^{-1}$ concentration (compact phenotype). In relation to the control C concentration in the fungus decreased from around 36% to 32% and from 39% to 36% for $0g.L^{-1}$ and $10g.L^{-1}$ glucose concentrations respectively, however the differences are not statistically significant. However, despite the decrease of the values of C accumulation, the pattern was similar to the control, so the proportion of C in the fungus tended to increase with the availability of glucose.

Stable isotope $\delta^{15}N$ and $\delta^{13}C$ discrimination

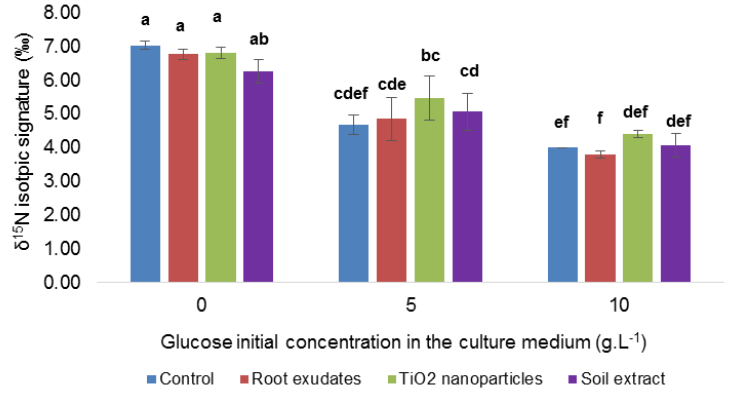


Figure 15.Stable $\delta^{15}N$ isotopic signatures of P. indica colonies subjected to root exudate (red bars), TiO_2 nanoparticles (green bars) and soil extract (purple bars) treatments at different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Letters above the bars indicate significant differences ($p<0.05$) accordingly to Tukey test. N=36.

Regarding $\delta^{15}N$ isotope, none of the treatments significantly influenced the $\delta^{15}N$ isotopic signature of the fungus (fig.15; table 5).

Thus, for $0g.L^{-1}$ of glucose, independently of the treatment, the fungus always has a higher value of $\delta^{15}N$ than the culture medium ($\delta^{15}N=5.2$). The values obtained denote the fungal discrimination for peptone and/or casein ($\delta^{15}N$ = 6.6 and 6.9 respectively) in preference to yeast extract ($\delta^{15}N$=-

0.1) as N sources (table 5). So, any of the treatments influenced the isotopic signature of *P. indica* compact phenotype.

For 5g.L^{-1} of glucose, the colonies subjected to the root exudates treatment showed similar isotopic signatures ($\delta^{15}N$=4.9) to the control and the culture medium ($\delta^{15}N$=4.6) which indicates that on this situation the fungus started to consume more yeast extract ($\delta^{15}N$=-0.1) as N source. For both nanoparticles and soil extract treatments the fungal $\delta^{15}N$ isotopic signatures (means $\delta^{15}N$=5.4 and $\delta^{15}N$=5.0, respectively) were higher than the control ($\delta^{15}N$=4.5), which indicates that the fungus incorporated lower amounts of yeast extract than the cultures treated with root exudates or control. However, the differences were not statistically significant.

For 10g.L^{-1}, independently of the treatment applied the fungus did not use any of the N sources preferentially, since the fungal isotopic signatures were not identical to neither of the isotopic signatures of the N sources available in the culture medium (peptone $\delta^{15}N$=6.6; casein $\delta^{15}N$=6.9; yeast extract $\delta^{15}N$=-0.1), and were also identical to the culture medium ($\delta^{15}N$=4.2), which indicates that the fungus is consuming all N components of the culture medium on the proportions they were present.

In summary, for 0g.L^{-1} and 10g.L^{-1} of glucose concentrations, the addition of root exudates, TiO_2 nanoparticles or soil extract to the culture medium did not alter the fungal isotopic signature. Thus, at 0g.L^{-1} of glucose situations the fungus consumed preferentially peptone and/or casein as N sources, whereas at 10g.L^{-1} of glucose did not used any of the N sources available (table 5).

Therefore, as in control cultures, the consumption of yeast extract increased with the glucose availability in the culture medium in all treatments.

Table 5. Stable $\delta^{15}N$ isotopic signatures of P. indica colonies subjected to root exudate, TiO2 nanoparticles and soil extract treatments at different glucose concentrations.

	$\delta^{15}N$ isotopic signature (‰) (mean ± **SD**)		
Treatments/ culture medium components	Glucose 0g.L^{-1} ($\delta^{15}N$=5.2)	Glucose 5g.L^{-1} ($\delta^{15}N$=4.6)	Glucose 10g.L^{-1} ($\delta^{15}N$=4.2)
Control	7.06 ± **0.12**	4.51 ± **0.02**	4.01 ± **0.01**
Root exudates	6.76 ± **0.12**	4.85 ± **0.41**	3.79 ± **0.08**
TiO_2 nanoparticles	6.83 ± **0.17**	5.45 ± **0.54**	4.40 ± **0.11**
Soil extract	6.25 ± **0.30**	5.07 ± **0.45**	4.08 ± **0.30**
Peptone	6.6	6.6	6.6
Casein	6.9	6.9	6.9
Yeast extract	-0.1	-0.1	-0.1
Glucose	------	------	------

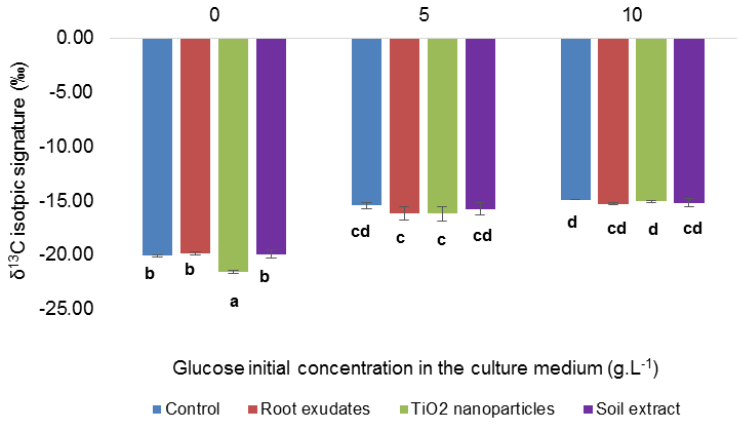


Figure 16 Stable $\delta^{13}C$ isotopic signatures of P. indica colonies subjected to root exudate (red circles), TiO_2 nanoparticles (green circles) and soil extract (purple circles) treatments at different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Letters above the bars indicate significant differences ($p<0.05$) accordingly to Tukey test. N=36.

Table 6. Stable $\delta^{13}C$ isotopic signatures of P. indica colonies subjected to root exudate, TiO2 nanoparticles and soil extract treatments at different glucose concentrations

	$\delta^{13}C$ isotopic signature (‰) (mean ± **SD**)		
Treatments/ Culture medium components	Glucose 0g.L^{-1} ($\delta^{13}C$=-21.7)	Glucose 5g.L^{-1} ($\delta^{13}C$=-16.0)	Glucose 10g.L^{-1} ($\delta^{13}C$=-14.4)
Control	-20.1 ± **0.1**	-15.6 ± **0.2**	-14.9 ± **0.1**
Root exudates	-19.8 ± **0.1**	-16.2 ± **0.4**	-15.3 ± **0.3**
TiO_2 nanoparticles	-21.6 ± **0.0**	-16.2 ± **0.5**	-15.1 ± **0.1**
Soil extract	-19.9 ± **0.1**	-15.8 ± **0.5**	-15.2 ± **0.1**
Peptone	-16.9	-16.9	-16.9
Casein	-26.8	-26.8	-26.8
Yeast extract	-25.5	-25.5	-25.5
Glucose	-12.1	-12.1	-12.1

About $\delta^{13}C$ isotope, neither the addition of root exudates nor soil extract affected the fungal $\delta^{13}C$ isotopic signature (fig.16). At 0g.L^{-1} of glucose condition, the treated cultures, as well as the control, showed a higher isotopic signature than the culture medium, that probably corresponds to the signature of the sources that the fungus used in N nutrition, peptone and casein ($\delta^{13}C$=-16.9 and -26.8 respectively). However, when subjected to the TiO_2 nanoparticles treatment, the fungal isotopic signature ($\delta^{13}C$=-21.6) was similar to the culture medium ($\delta^{13}C$=-21.7), which indicates that the fungus did not preferentially use any of the sources available in the culture medium in order to get C, i.e. it consumed all the organic sources available in the culture medium (peptone, casein and yeast extract) as they were present in the culture medium. Interestingly, these results suggest that when the fungus is subjected to the presence of TiO_2 and to the absence of glucose it only used the yeast extract to incorporate C, but it excluded the N present on that organic source.

When glucose was present in the culture medium (5 and 10g.L^{-1}) neither of the treatments altered significantly the fungal $\delta^{13}C$ isotopic signatures, so the pattern was maintained similar to the

control, in which the fungus did not discriminate for any of the sources of C, i.e. it did not incorporate any of the C sources preferentially (table 6).

Final pH

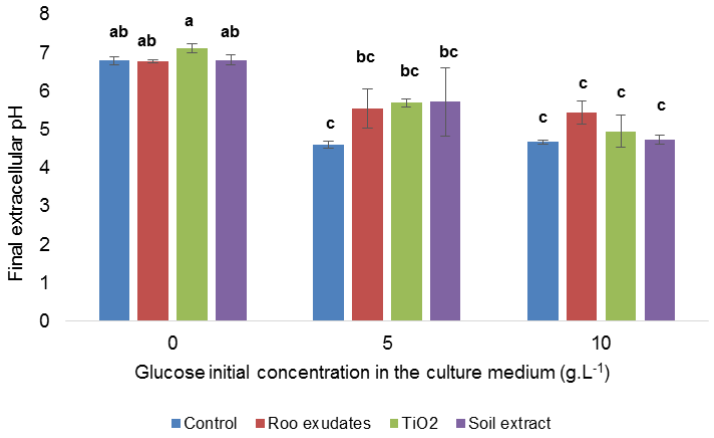


Figure 16. Final extracellular pH of 11 days P. indica cultures subjected to root exudate (red bars), TiO_2 nanoparticles (green bars) and soil extract (purple bars) treatments at different glucose concentrations. The concentrations of the other components of the culture medium were maintained as the original. Letters above the bars indicate significant differences ($p<0.05$) accordingly to Tukey test. N=36.

Regarding final pH of the culture medium, all three treatments follow the same pattern as the control, where the extracellular pH is lower in the presence of glucose (fig.16). However, the decrease was not as pronounced as in the control, which is mainly reflected in the cultures with 5g.L^{-1} of glucose, wherein the extracellular pH of the cultures subjected to the different treatments tended to be higher than the control (table 6).

Table 6.Final extracellular pH of 11 days P. indica cultures subjected to root exudate, TiO_2 nanoparticles and soil extract treatments at different glucose concentrations

	Final extracellular pH (mean ± **SD**)		
Treatments	Glucose 0g.L^{-1}	Glucose 5g.L^{-1}	Glucose 10g.L^{-1}
Control	6.8 ± **0.1**	4.6 ± **0.09**	4.68 ± **0.05**
Root exudates	6.79 ± **0.04**	5.54 ± **0.51**	5.45 ± **0.30**
TiO_2 nanoparticles	7.13 ± **0.12**	5.7 ± **0.11**	4.96 ± **0.43**
Soil extract	6.81 ± **0.13**	5.73 ± **0.89**	4.74 ± **0.11**

Decomposition Potential and enzymatic activities

Concerning C, N and P decomposition potentials, the effects of root exudates, TiO_2 nanoparticles and soil extract treatments were only visible in the compact phenotype (0g.L^{-1} glucose) (fig.18). Thus, regarding decomposition potential of the organic compounds (C decomposing potential), the addition of nanoparticles to the culture medium without glucose led to a decrease of the enzymatic activities related with C decomposition, comparing to the control cultures (from 168.27

to 97.23µmol.h^{-1}.g^{-1} of mycelium). Also the addition of root exudates and soil extract to the culture medium tend to decrease C decomposing potential of the compact phenotype (130.05 and 123.57µmol.h^{-1}.g^{-1} of mycelium, respectively), although the differences were not statistically significant. However, in the explorer phenotype neither of the treatments affected the fungal C decomposing potential.

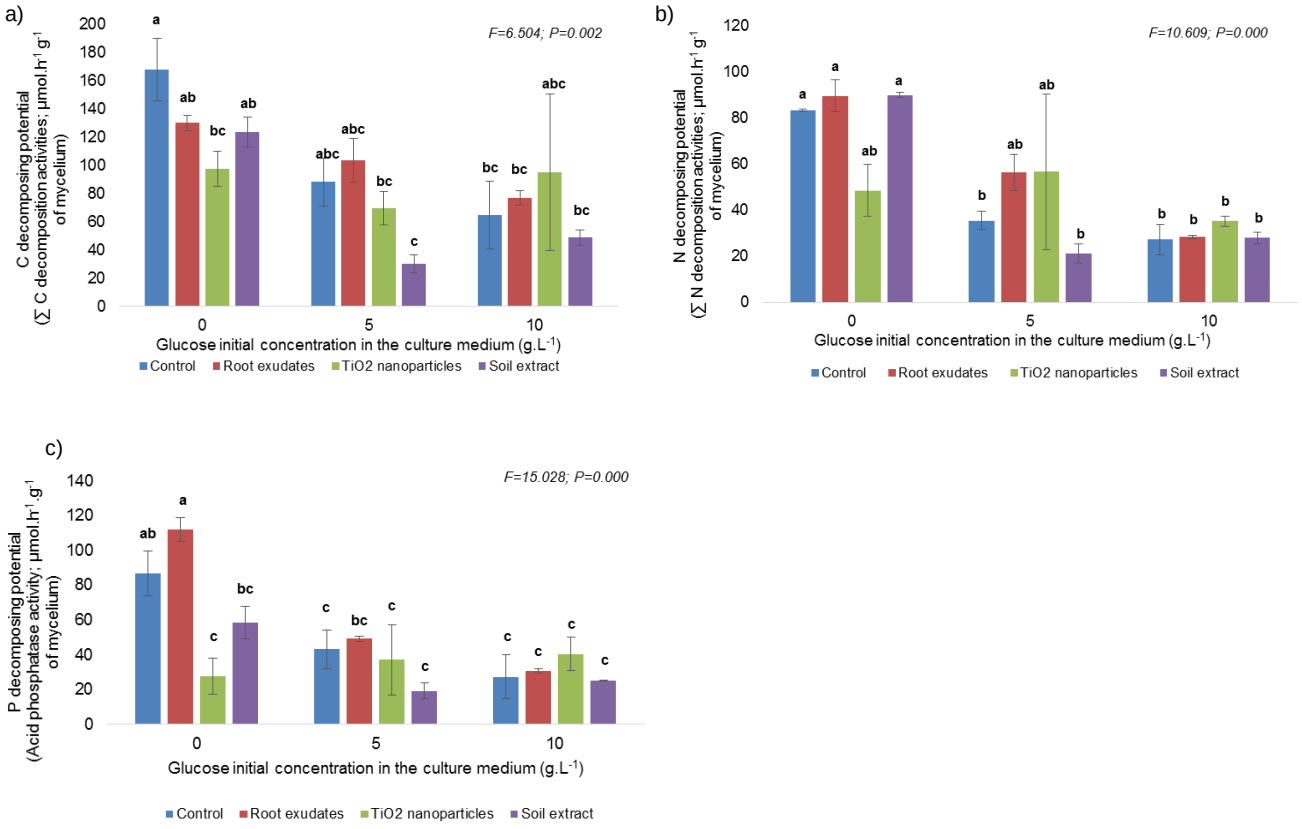


Figure 18.C, N and decomposition potential of P. indica cultures (a), b) and c) respectively) subjected to root exudates (red bars) TiO_2 nanoparticles (green bars) and soil extract (purple bars) treatments. ∑C decomposition: β-xylosidase, cellobiohydrolase, β-glucuronidase and β.glucosidase activities; ∑N decomposition: Leucine aminopeptidase and N-acetyl-glucosaminidase activities. Letters above the bars indicate significant differences (p<0.05) accordingly to Tukey test. N=24.

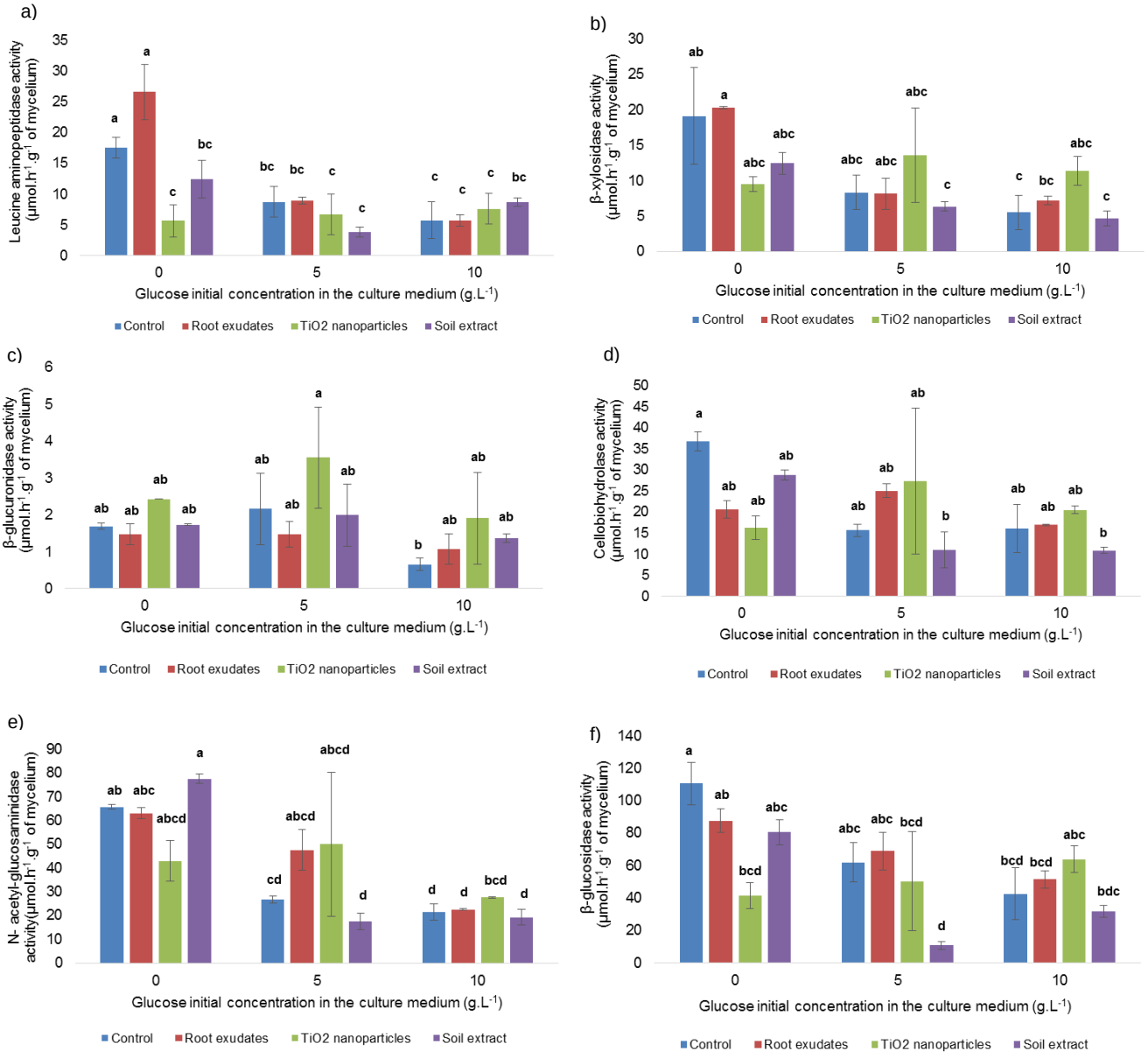
These results suggest that on low C/N conditions the addition of root exudates, TiO_2 or soil extract to the culture medium led to a decrease of the C decomposing potential of *P. indica* cultures.

Concerning N decomposition potential, neither of the phenotypes were affected by any of the treatments, but there was a strong trend for the addition of TiO_2 to decrease N decomposing potential in the compact phenotype (decreased from 83.24 to 48.54µmol.h^{-1}.g^{-1} of mycelium) (fig. 18).

These results suggest that neither roots exudates nor soil extract treatments altered the N decomposition potential of the fungus among the different C/N conditions, but the addition of TiO_2 led to a loss in the N decomposition ability of the compact phenotype.

About P decomposition potential, again there were only significant differences on the compact phenotype in response to the different treatments. Hence, the addition on root exudates led to a trend towards an increase of phosphatase activity of the fungus from 86.96 to 112.10µmol.h^{-1}.g^{-1} of mycelium (although the differences were not statistically significant). Cultures without glucose and treated with TiO_2 nanoparticles (40.40µmol.h^{-1}.g^{-1} of mycelium) and soil extract (58.40µmol.h^{-1}.g^{-1} of mycelium) showed lower P decomposing potentials than control cultures, but the differences were only significant for TiO_2 treated cultures.

These results suggest that root exudate treatment improved P decomposing potential of the fungus when glucose was absent from the culture medium, but both soil extract and TiO_2 had a negative impact in acid phosphatase activity, for compact phenotype.



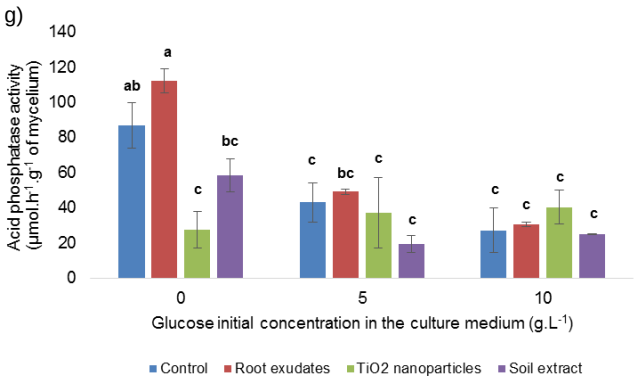


Figure 19.Potential enzymatic activities of P. indica cultures subjected root exudates (red bars), TiO_2 nanoparticles (green bars) and soil extract (purple bars) treatments at different glucose concentrations. The cultures with 0g.L^{-1} of glucose corresponds to compact phenotype; 5g.L^{-1} Compact & Explorer phenotype; 10g.L^{-1} Explorer phenotype. a) Leucine aminopeptidase; b) β-xylosidase; c) β-glucuronidase ; d) Cellobiohydrolase; e) N-acetyl-glucosaminidase; f) β-glucosidase; g) Acid phosphatase. Letters above the bars indicate significant differences (p<0.05) accordingly to Tukey test. N=24.

Regarding the potential enzymatic activity assays, the pattern observed in the cultures subjected to the root exudates and soil extract treatments remained similar to the control cultures, in which the cultures without glucose had a higher decomposition potential than cultures where 10g.L^{-1} of glucose was added, excepting for β-glucuronidase and cellobiohydrolase enzymes (fig.19). However, for cultures treated with TiO_2 nanoparticles this pattern was altered, and no differences were detected among the different glucose concentrations (i.e. among the fungal phenotypes) for every enzymes tested.

Although the patterns were similar, the individual enzymatic profile of the cultures subjected to the different treatments differ quantitatively from the control. Thus, the main changes were observed when glucose was absent from the culture medium, mainly by the increase of leucine aminopeptidase and phosphatase activities (from 17.5 to 26.6 and 86.96 to 112.10µmol.h^{-1}.g^{-1} of mycelium respectively) and the decrease of β-glucosidase activity (from 110.60 to 87.59µmol.h^{-1}.g^{-1} of mycelium) in cultures subjected to root exudates treatment. The activity of cellobiohydrolase were also affected (decreased from 36.84 to 20.65µmol.h^{-1}.g^{-1} of mycelium).

For 5g.L^{-1} of glucose added to the culture medium, the activities of cellobiohydrolase and N-acetyl-glucosaminidase tended to be higher than the control although the differences were not statistically significant.

The addition of TiO_2 nanoparticles to the culture medium completely changed the physiological profile of *P. indica* colonies.TiO_2 application led to a decrease of the activities of all extracellular hydrolytic enzymes in glucose free culture medium, in comparison to the TiO_2 free control treatment. These activities were similar to the control cultures with 5g.L^{-1} of glucose.

The addition of soil extract to culture medium with 0g.L^{-1} of glucose tended to stimulate the activity of N-acetyl-glucosaminidase (from 65.70 to 77.59µmol.h^{-1}.g^{-1} of mycelium), although the differences were not statistically significant. The activities of β-xylosidase, cellobiohydrolase, β-glucosidase and acid phosphatase tended to decrease in response to soil extract treatment when

comparing to the control, although the differences were not statistically significant. The activity of leucine-aminopeptidase decreased in 0g.L^{-1} of glucose cultures treated with soil extract (from 17.53 to 12.44µmol.h^{-1}.g^{-1} of mycelium) and β-glucuronidase were not affected at 0g.L^{-1} glucose concentrations, in comparison to control cultures.

In culture medium with 5g.L^{-1} of glucose, all the enzymatic activities were similar to the control, except for β-glucosidase which was severely affected (decreased from 62.03 to 10.83µmol.h^{-1}.g^{-1} of mycelium).

Regarding culture medium with 10g.L^{-1} of glucose, the decomposition potential of *P. indica* was not affected by adding soil extract to the cultures.

Discussion

The effect of root exudates treatment on P. indica physiology

The addition of root exudates did not have any impact on the percentages of C and N in mycelium, nor in the isotopic signatures of both *P. indica* phenotypes (figs.13 to 16).

Also, the extracellular pH of the cultures was not affected by the addition of root exudates (fig. 17). Therefore, the alkalinisation of glucose free culture medium is due to the release of ammonia from cells, resulting from the use of aminoacids as C sources[49]. The acidification of extracellular pH in cultures with glucose (explorer phenotype) is due to the release of organic acids resulting from C catabolism processes[45].

Regarding the decomposition potential of *P. indica,* the physiological profile of the explorer phenotype was not altered by the presence of root exudates. However, the 0g.L^{-1} of glucose cultures (compact phenotype) showed a different enzymatic profile when compared to the control (fig.19). The activities of leucine aminopeptidase and acid phosphatase increased, whereas the activities of cellobiohydrolase and β-glucosidase decreased in response to root exudates. These results partially contradict our hypothesis, since it was expected a stimulation of the enzymes involved in the colonization process, namely cellobiohydrolase. Yet, the stimulation of such enzymes related to the colonization process mainly occur during the implementation of colonization and locally, so that the effect of root exudates was not visible in all mycelium.

Nevertheless our results are in agreement with other studies where root exudation enhanced the microbial activity related to the breakdown of organic N in subalpine coniferous forest and stimulated the acid phosphatase activity in temperate forest soils[72] [73].

The stimulation of acid phosphatase activity (fig.20) is in agreement with the literature, since phosphatase activity is described to be positively correlated with N availability (in relative P-limited systems) [74]. Also some studies have demonstrated enhanced N cycling in the vicinity of plant roots [75].

The addition of root exudates also led to a decrease in decomposition potential of organic compounds, mainly by the decreased cellobiohydrolase and β-glucosidase activities. These results reveal that when the fungus was exposed to root exudates, the combined action of the enzymes related to C release is repressed. Such may happen probably because in root exudates there were more simple C compounds and other plant-signalling molecules that modulate the fungal physiology to a pre-symbiotic stage. Previous studies revealed that root exudates stimulate glucose uptake and hyphal branching of *G. intraradices* spores, and stimulate the H^+ efflux and the H^+-ATPase activity of the fungal plasma membrane of germinating spores of *Gigaspora margarita*. This supports the hypothesis during pre-symbiotic a stimulus enhances the incorporation of simple C compounds over complex organic compounds by fungi [76] [77].

The effect of TiO_2 nanoparticles treatment on P. indica physiology

Although the effect of TiO_2 nanoparticles on *P. indica* morphology and biomass production has been described previously by Suman *et al*. (2010), suggesting an improvement of fungal performance, a physiological approach about the effect of these nanomaterials has never been done[11].

On this chapter, the physiological characterization of both compact and explorer phenotypes of *P. indica* in response to the presence of TiO_2 nanoparticles was addressed. The addition of TiO_2 nanoparticles to the culture medium did not change *P. indica* phenotypes, comparing to the control cultures, which contradict the results of Suman *et al*. (2010) where a compact phenotype was exhibited in $10g.L^{-1}$ of glucose medium cultures.

On compact phenotype cultures (i.e. grown with $0g.L^{-1}$ of glucose) the addition of TiO_2 significantly affected the percentages of both N and C on fungal biomass (although the differences were statistically significant in N percentage) (figs. 13 and 14). These results are consistent with the decomposition potential results, where activity of the extracellular enzymes was severely affected by the presence of TiO_2 (fig.18). Therefore, the addition of TiO_2 nanoparticles to the culture medium led to a decrease of the activities of the enzymes involved on the degradation of organic compounds present in the culture medium (peptone, casein and yeast extract) resulting in the decrease of the N and C proportions in the fungus. So the stimulus from the lack of glucose on the decomposition potential of *P. indica* compact phenotype is lost when TiO_2 nanoparticles are added to the culture medium. For the same conditions, the fungal $\delta^{15}N$ isotopic signature did not change when compared with the control, where the fungus showed a preference for peptone and casein as N sources (fig.15). However, the $\delta^{13}C$ isotopic signature results showed that on the contrary to what occurred in the control cultures, the fungus consumed all the organic sources, peptone, casein and yeast extract in order to get C (fig.16). These results indicate that when glucose is absent from the culture medium, the fungus uses the C present on yeast extract but rejects the N prevenient from this source. Previous studies revealed that TiO_2 nanoparticles stimulate the metabolic flux of glycolysis pathway in *E. coli*, *B. subtilis* and *S. cerevisiae* [68]. Thus, the use of C present in yeast extract by the fungus (when glucose was absent from the culture

medium) can be explained by the need to assimilate more C into cells due to the higher rate of glycolysis.

The addition of TiO_2 nanoparticles on $10g.L^{-1}$ cultures did not change *P. indica* morphology as described by Suman *et al*. (2010). Hence, $10g.L^{-1}$ of glucose cultures still exhibited the explorer phenotype (instead of the compact phenotype). Similarly, neither both N and C percentages in fungal biomass nor $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures were affected in explorer phenotype (figs.13 to 16).

Also, the decomposition potential of *P. indica* explorer phenotype did not change in response to TiO_2 nanoparticles treatment (figs.18 and 19).

Therefore, we can conclude that TiO_2 treatment had a harmful effect on the cultures grown without glucose and did not affect the normal physiology of *P. indica* explorer phenotype ($10g.L^{-1}$ of glucose), unlike what is described by Suman *et al*. (2010). Thus, the promoting effect of TiO_2 nanoparticles treated *P. indica* culture filtrate in plants might be due to the release other plant growth promoting molecules and not directly by the enhancement of extracellular enzymatic activities.

The effect of soil extract treatment on P. indica physiology

The addition of soil extract did not have any impact on the proportions of C and N, nor in the isotopic signatures on any of the *P. indica* phenotypes (figs.13 to 16).

Concerning the decomposition potential, the addition of soil extract did not affect the enzymatic profile of *P. indica* explorer phenotype (fig.18).

However, when the fungus was grown only with complex organic sources ($0g.L^{-1}$of glucose, resulting in compact phenotype), the activities of cellobiohydrolase, β-glucosidase and acid phosphatase enzymes tended to decrease, while N-acetyl-glucosaminidase activity was enhanced (but the differences were not statistically significant) (fig.19). Also, the activity of leucine aminopeptidase was lower when comparing with control cultures (fig.19). These results can be possibly explained by the presence of simple organic compounds in the soil extract. Thus, having simpler organic sources available, the fungus failed to produce enzymes responsible for the degradation of complex organic compounds. However, since those compounds are available at low concentrations when compared to the organic sources from the culture medium (the volume of soil extract corresponds to only 10% of the final volume of the culture medium) the fungus still consumed peptone and casein from the culture medium, as revealed by the fungal isotopic signatures (figs. 15 and 16). Our results are in agreement with the study performed by Fan *et al.* (2012) where the presence of soil extract repressed genes involved on carbohydrates metabolism of *B. amyloliquefaciens* FZB42 strains.[65]

These results showed that soil extract only affects the potential decomposition activities of the fungus when glucose is absent from the culture medium, probably due to the presence of additional organic compounds that are consumed by the fungus in order to get C, N and P.

Conclusion

In this study we analysed the influence of root exudates, TiO_2 and soil extract in the physiology of both phenotypes of *P. indica* previously characterized. Neither of the treatments changed the normal morphology of the fungus, and the differences on fungal physiology were only visible when glucose was absent from the culture medium (compact phenotype).

The presence of root exudates (which simulates the presence of a host plant and possibly stimulates the switch from free living to pre-symbiotic stage of the fungus), enhances the decomposing activity of the fungus. Such happened in the absence of glucose (compact phenotype), with a more visible effect on N and P related enzymes than on organic compounds hydrolysing enzymes.

On the other hand, the addition of TiO_2 nanoparticles to the culture medium repressed fungal performance on the decomposition of the organic matter when it was grown without glucose (compact phenotype) and did not alter the decomposition potential of explorer phenotype.

Nevertheless, TiO_2 nanoparticles stimulated the consumption of C from yeast extract, when glucose was not available.

Concerning the presence of soil extract in the production of inoculum, it resulted in decreased enzymatic activities of enzymes involved in the degradation of organic matter in the compact phenotype ($0g.L^{-1}$ of glucose), but did not alter the physiological profile of explorer phenotype ($10g.L^{-1}$ of glucose).

From this experiment we can conclude that the major modulator of *P. indica* physiology was the C to N ratio, and the effects of root exudates, TiO_2 nanoparticles and soil extract were amplified when glucose was absent from culture medium (low C/N ratio). The effects on fungal decomposition potential were only beneficial in response to root exudates treatment.

Final remarks

On this study we characterized morphologically and physiologically two phenotypes of the fungus *P. indica* (compact and explorer phenotypes). The occurrence of each phenotype is regulated by the proportions of C to N concentrations in the culture medium and independent of the C source (glucose or sucrose).

Our results revealed that the compact phenotype has a higher decomposition potential (necrotrophic lifestyle) than explorer phenotype (biotrophic lifestyle).

The application of *P. indica* inocula in maize and barley roots did not exerted a beneficial effect on plant biomass, probably due to the nutrient rich nutrition that the plants were subjected to.

Considering the decomposition potential of colonized roots, due to the heterogeneity of the data (that reflect the different levels of root colonization) there were no significant differences among *P. indica* phenotypes in the different hosts.

The addition of root exudates, TiO_2 nanoparticles and soil extract only exerted effect on *P. indica* compact phenotype (low C/N conditions), whereas root exudates particularly stimulated the fungal N and P decomposition potentials. Soil extract and TiO_2 treatments led to a decrease of the enzymatic activities of *P. indica* compact phenotype and did not alter the normal decomposition potential of explorer phenotype.

Considering these results, it can be concluded that the major modulator of the fungus physiology was the C/N proportions. On C low availability conditions, the fungus has a higher N and P decomposing potential when under the influence of root exudates from a host plant then when subjected to bulk soil extract.

Further studies are needed in order to test the different *P. indica* phenotypes in more extreme conditions (i.e. low nutrition, abiotic stress, etc.), where the hosts may effectively benefit of the potential of each phenotype. Such studies should be performed in order to evaluate if the characteristics acquired *in vitro* persist on field conditions.

References

1. Verma, S. *et al. Piriformospora indica* , gen . et sp . nov ., a new root-colonizing fungus. *Mycologia* **5,** 896–903 (1998).

2. Oelmüller, R., Sherameti, I., Tripathi, S. & Varma, A. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis* **49,** 1–17 (2009).

3. Baltruschat, H. *et al.* Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytol.* **180,** 501–10 (2008).

4. Achatz, B. *et al.* Root colonization by *Piriformospora indica* enhances grain yield in barley under diverse nutrient regimes by accelerating plant development. *Plant Soil* **333,** 59–70 (2010).

5. Waller, F. *et al.* The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 13386–91 (2005).

6. Kumar, M., Yadav, V., Tuteja, N. & Johri, A. K. Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. *Microbiology* **155,** 780–90 (2009).

7. Bagde, U. S., Prasad, R. & Varma, A. Interaction of Mycobiont : *Piriformospora indica* with Medicinal plants and plants of Economic importance. *African J. Biotechonoly* **9,** 9214–9226 (2010).

8. Rai, M., Acharya, D., Singh, A. & Varma, A. Positive growth responses of the medicinal plants Spilanthes calva and Withania somnifera to inoculation by *Piriformospora indica* in a field trial. *Mycorrhiza* **11,** 123–128 (2001).

9. Kumar, V., Rajauria, G., Sahai, V. & Bisaria, V. S. Culture filtrate of root endophytic fungus *Piriformospora indica* promotes the growth and lignan production of Linum album hairy root cultures. *Process Biochem.* **47,** 901–907 (2012).

10. Varma, A. *et al.* in *Mycota IX* (Hock, B.) 125–150 (Springer Berlin Heidelberg, 2001).

11. Suman, Prasad, R., Jain, V. K. & Varma, A. Role of nanomaterials in symbiotic fungus growth enhancement. *Curr. Sci.* **99,** 1189–1192 (2010).

12. Lahrmann, U. *et al.* Host-related metabolic cues affect colonization strategies of a root endophyte. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 13965–70 (2013).

13. Alexopoulos, C. J., Mims, C. W. & Blackwell, M. *Introductory Mycology*. (Wiley, 1996).

14. Berendsen, R. L., Pieterse, C. M. J. & Bakker, P. a H. M. The rhizosphere microbiome and plant health. *Trends Plant Sci.* **17,** 478–86 (2012).

15. Smith, S. E. & Read, D. J. *Mycorrhizal symbiosis*. (Academic Press, 1997).

16. Broeckling, C. D., Broz, A. K., Bergelson, J., Manter, D. K. & Vivanco, J. M. Root exudates regulate soil fungal community composition and diversity. *Appl. Environ. Microbiol.* **74,** 738–44 (2008).

17. Hirsch, A. M. *et al.* Molecular signals and receptors: controlling rhizosphere interacions between plants and other organisms. *Ecology* **84,** 858–868 (2003).

18. Varma, A., Bakshi, M., Lou, B., Hartmann, A. & Oelmueller, R. *Piriformospora indica*: A Novel Plant Growth-Promoting Mycorrhizal Fungus. *Agric. Res.* **1,** 117–131 (2012).

19. Kost, G. & Rexer, K. in *Piriformospora indica Sebacinales Their Biotechnol. Appl.* (Varma, A., Kost, G. & Oelmüller, R.) **33,** 25–36 (Springer Berlin Heidelberg, 2013).

20. Sun, C. *et al. Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *J. Plant Physiol.* **167,** 1009–17 (2010).

21. Varma, a *et al. Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Appl. Environ. Microbiol.* **65,** 2741–4 (1999).

22. Deshmukh, S. *et al.* The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 18450–7 (2006).

23. Taiz, L. & Zeiger, E. *Plant physiology*. (Sinauer Associates, 2002).

24. Qiang, X., Weiss, M., Kogel, K. & Shäfer, P. *Piriformospora indica* — a mutualistic basidiomycete with an exceptionally large plant host range. *Mol. Plant* **13,** 508–518 (2012).

25. Zuccaro, A. *et al.* Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. *PLoS Pathog.* **7,** e1002290 (2011).

26. Lorenz, M. C. & Heitman, J. The MEP2 ammonium permease regulates pseudohyphal differentiation in Saccharomyces cerevisiae. *EMBO J.* **17,** 1236–47 (1998).

27. Hilbert, M. *et al.* Indole derivative production by the root endophyte *Piriformospora indica* is not required for growth promotion but for biotrophic colonization of barley roots. *New Phytol.* **196,** 520–34 (2012).

28. Farinas, C. *A Parede Celular Vegetal E As Enzimas Envolvidas Na Sua Degradação*. (2011).

29. Dodd, D. & Cann, I. K. O. Enzymatic deconstruction of xylan for biofuel productin. *Glob. Chang. Biol. Bioenergy* **1,** 2–17 (2009).

30. Ramstedt, M. & Söderhäll, K. Protease, phenoloxidase and pectinase activities in mycorrhizal fungi. *Trans. Br. Mycol. Soc.* **81,** 157–161 (1983).

31. Burke, R. M. & Cairney, J. W. Laccases and other polyphenol oxidases in ecto- and ericoid mycorrhizal fungi. *Mycorrhiza* **12,** 105–116 (2002).

32. Basiewicz, M. Molecular and phenotypic characterization of endophytic Sebacinoid strains. (2010).

33. Basiewicz, M. *et al.* Molecular and phenotypic characterization of Sebacina vermifera strains associated with orchids, and the description of Piriformospora williamsii sp. nov. *Fungal Biol.* **116,** 204–13 (2012).

34. Acosta-Martínez, V. & Tabatabai, M. . Arylamidase activity in soils: effect of trace elements and relationships to soil properties and activities of amidohydrolases. *Soil Biol. Biochem.* **33,** 17–23 (2001).

35. Ladd, J. . & Butler, J. H. . Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates. *Soil Biol. Biochem.* **4,** 19–30 (1972).

36. Divon, H. H. & Fluhr, R. Nutrition acquisition strategies during fungal infection of plants. *FEMS Microbiol. Lett.* **266,** 65–74 (2007).

37. Kajala, K. *et al.* Strategies for engineering a two-celled C(4) photosynthetic pathway into rice. *J. Exp. Bot.* **62,** 3001–10 (2011).

38. Pritsch, K. *et al.* Optimized assay and storage conditions for enzyme activity profiling of ectomycorrhizae. *Mycorrhiza* **21,** 589–600 (2011).

39. Kumar, V., Sahai, V. & Bisaria, V. S. High-density spore production of *Piriformospora indica*, a plant growth-promoting endophyte, by optimization of nutritional and cultural parameters. *Bioresour. Technol.* **102,** 3169–75 (2011).

40. Singh, A., Singh, A., Kumari, M., Rai, M. K. & Varma, A. Biotechnological Importance of *Piriformospora indica* Verma et al-A Symbiotic Mycorrhiza-like Fungus : An Overview Novel. *Indian J. biotecnhology* **2,** 65–75 (2003).

41. Lorenz, M. C. & Heitman, J. The MEP2 ammonium permease regulates pseudohyphal differentiation in Saccharomyces cerevisiae. *EMBO J.* **17,** 1236–47 (1998).

42. Lorenz, M. C. *et al.* The G protein-coupled receptor gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in Saccharomyces cerevisiae. *Genetics* **154,** 609–22 (2000).

43. Biswas, S., Van Dijck, P. & Datta, A. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of Candida albicans. *Microbiol. Mol. Biol. Rev.* **71,** 348–76 (2007).

44. Bertram, P. G. *et al.* Convergence of TOR-Nitrogen and Snf1-Glucose Signaling Pathways onto Gln3. *Mol. Cell. Biol.* **22,** 1246–1252 (2002).

45. Orij, R., Postmus, J., Ter Beek, A., Brul, S. & Smits, G. J. In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in Saccharomyces cerevisiae reveals a relation between intracellular pH and growth. *Microbiology* **155,** 268–78 (2009).

46. Martínez-Muñoz, G. & Kane, P. Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. *J. Biol. Chem.* **283,** 20309–20319 (2008).

47. Kresnowati, M., Suarez-Mendez C, Groothuizen, M., van Winden, W. & Heijnen, J. Measurement of fast dynamic intracellular pH in Saccharomyces cerevisiae using benzoic acid pulse. *Biotechnol. Bioeng.* **97,** 86–98 (2007).

48. Ramos, S., Balbín, M., Raposo, M., Valle, E. & Pardo, L. a. The mechanism of intracellular acidification induced by glucose in Saccharomyces cerevisiae. *J. Gen. Microbiol.* **135,** 2413–22 (1989).

49. Vylkova, S. *et al.* The Fungal Pathogen Candida albicans Autoinduces Hyphal Morphogenesis by Raising Extracellular pH. *MBio* **2,** (2011).

50. Kalembasa, S. & Symanowicz, B. Enzymatic Activity of Soil after Applying Various Waste Organic Materials , Ash , and Mineral Fertilizers. *Polish J. Environ. Stud.* **21,** 1635–1641 (2012).

51. Lopes, A. A. de C. *et al.* Interpretation of Microbial Soil Indicators as a Function of Crop Yield and Organic Carbon. *Soil Sci. Socitety Am. J.* **77,** 461–472 (2012).

52. Kumari, R., Kishan, H., Bhoon, Y. K. & Varma, A. Colonization of cruciferous plants by *Piriformospora indica*. *Curr. Sci.* **85,** 1672–1674 (2003).

53. Dakora, F. D. & Phillips, D. A. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant Soil* **245,** 35–47 (2002).

54. Horst, R. J. *et al.* A model of Ustilago maydis leaf tumor metabolism. *Plant Signal. Behav.* **5,** 1446–9 (2010).

55. Tavernier, V. *et al.* The plant nitrogen mobilization promoted by Colletotrichum lindemuthianum in Phaseolus leaves depends on fungus pathogenicity. *J. Exp. Bot.* **58,** 3351–60 (2007).

56. Gowik, U. & Westhoff, P. The path from C3 to C4 photosynthesis. *Plant Physiol.* **155,** 56–63 (2011).

57. Neumann, G. & Römheld, V. in *Rhizosph. Biochem. Org. Subst. soil-plant interface* (Pinton, R., Varanini, Z. & Nannipieri, P.) 41–93 (Marcel Dekker, 2000).

58. Uren, N. in *Rhizosph. Biochem. Org. Subst. soil-plant interface* (Pinton, R., Varanini, Z. & Nannipieri, P.) 19–40 (Marcel Dekker, 2000).

59. Bais, H. P. A. L., Loyola-vargas, V. M., Flores, H. E. & Vivanco, A. N. D. J. M. Root-specific metabolism: The biology and biochemistry of underground organs. *Vitr. Cell. Dev. Biol. - Plant* **37,** 730–741 (2001).

60. Badri, D. V. & Vivanco, J. M. Regulation and function of root exudates. *Plant. Cell Environ.* **32,** 666–681 (2009).

61. Walker, T. S., Bais, H. P., Grotewold, E. & Vivanco, J. M. Update on Root Exudation and Rhizosphere Biology Root Exudation and Rhizosphere Biology 1. *Plant Physiol.* **132,** 44–51 (2003).

62. Perret, X., Staehelin, C. & Broughton, W. J. Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* **64,** 180–201 (2000).

63. Bücking, H. *et al.* Root exudates stimulate the uptake and metabolism of organic carbon in germinating spores of Glomus intraradices. *New Phytol.* **180,** 684–95 (2008).

64. Besserer, A. *et al.* Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. *PLoS Biol.* **4,** e226 (2006).

65. Fan, B. *et al.* Transcriptomic profiling of Bacillus amyloliquefaciens FZB42 in response to maize root exudates. *BMC Microbiol.* **12,** 116 (2012).

66. Guzman, K., Taylor, M. & Banfield, J. F. Environmental risks of nanotechnology: National Nanotechnology Initiative funding 2000-2004. *Environ. Sci. Technol.* **40,** 1401–1407 (2006).

67. Moghadam, B. Y., Hou, W.-C., Corredor, C., Westerhoff, P. & Posner, J. D. Role of nanoparticle surface functionality in the disruption of model cell membranes. *Langmuir* **28,** 16318–26 (2012).

68. Park, S. *et al.* Toxic effects of titanium dioxide nanoparticles on microbial activity and metabolic flux. *Biotechnol. Bioprocess Eng.* **17,** 276–282 (2012).

69. Gelsomino, A., Keijzer-wolters, A., Cacco, G. & van Elsas, J. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturating gradient gel eletrophoresis. *J. Microbiol. Methods* **38,** 1–15 (1999).

70. Carelli, M. *et al.* Genetic Diversity and Dynamics of Sinorhizobium meliloti Populations Nodulating Different Alfalfa Cultivars in Italian Soils Genetic Diversity and Dynamics of Sinorhizobium meliloti Populations Nodulating Different Alfalfa Cultivars in Italian Soils. *Appl. Environ. Microbiol.* **66,** 4785–4789 (2000).

71. Foster, R. . Microenvironments of soil microorganims. *Biol. Fertil. Soils* **1,** 189–203 (1988).

72. Brzostek, E. R., Greco, A., Drake, J. E. & Finzi, A. C. Root carbon inputs to the rhizosphere stimulate extracellular enzyme activity and increase nitrogen availability in temperate forest soils. *Biogeochemistry* **115,** 65–76 (2012).

73. Huajung, Y. *et al.* Enhanced root exudation stimulates soil nitrogen transformations in a subalpine coniferous forest under experimental warming. *Glob. Chang. Biol.* **19,** 2158–2167 (2013).

74. Treseder, K. K. & Vitousek, P. M. Effects of soil nutrient availability on investment in acquisition of N and P in Hawaiian rain forests. *Ecology* **82,** 946–954 (2001).

75. Jackson, L. E., Burger, M. & Cavagnaro, T. R. Roots, Nitrogen transformation and Ecosystem services. *Annu. Rev. Plant Biol.* **59,** 341–363 (2008).

76. Bücking, H. *et al.* Root exudates stimulate the uptake and metabolism of organic carbon in germinating spores of Glomus intraradices. *New Phytol.* **180,** 684–95 (2008).

77. Ramos, A. C., Façanha, A. R. & Feijó, J. a. Proton (H+) flux signature for the presymbiotic development of the arbuscular mycorrhizal fungi. *New Phytol.* **178,** 177–88 (2008).